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The Biology of *Zymomonas*

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INTRODUCTION

The alcoholic beverages in the Western world, such as beer, wine, champagne, etc., are usually made by fermentation with yeasts. The most commonly used organisms are strains of *Saccharomyces cerevisiae*. In many tropical areas of America, Africa, and Asia, other types of alcoholic beverages are very popular and widely used; these consist of plant saps undergoing a mixed fermentation, containing bacteria from the genus *Zymomonas*. In Europe, these bacteria occasionally grow in and spoil beer, fermented apple juice (cider), and pear juice (perry).

In the early 1950s, the genus *Zymomonas* acquired a certain fame among biochemists by the discovery of Gibbs and DeMoss (see below) that the anaerobic catabolism of glucose follows the Entner-Doudoroff mechanism. This was very surprising, since *Zymomonas* was the first example (and still is one of the very few) of an anaerobic organism using a pathway occurring mainly in strictly aerobic bacteria (85). This biochemical pathway in a gram-negative, nonsporeforming, polarly flagellated bacterial rod suggested the aerobic *Pseudomonadaceae* as possible remote relatives of *Zymomonas*, notwithstanding its largely anaerobic way of life.

In spite of its extensive use in many parts of the world, its great social implications as an ethanol producer, and its unique biochemical position, *Zymomonas* has not been studied extensively. Part of the available information has been very briefly reviewed by Dadds and Martin (50).

We collected most of the available strains from this genus and isolated several new ones. For the last 4 years, we have examined some 40-odd strains from Zaïre, Mexico, Indonesia, and Britain by modern taxonomical methods: deoxyribonucleic acid (DNA) base composition, DNA genome size, and genome similarity (163), protein electropherograms (164), and numerical analysis of numerous phenotypic features (50). In the present contribution we shall integrate all available literature data with our own experience and attempt to present a critical and coherent monograph of the biology of the genus *Zymomonas*. We shall not attempt to review here the technological aspects of these bacteria, except where they are helpful in understanding the biology.

The results of modern relatedness analysis in this genus, together with a critical examination of the literature data, show that all strains of *Zymomonas* constitute but one species, *Z. mobilis*, with two subspecies, *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae*. All strains in our collection, except three, and most strains from the literature belong in the former subspecies. Three strains from our collection and a few strains described in the literature belong to the subspecies *pomaceae*. The latter strains induce typical cider sickness symptoms. We consider this nomenclature as the only realistic one (50). On several occasions, we still have to use previous nomenclatures for a variety of reasons. A synonymy Table 7 is provided for the reader's convenience.

OCCURRENCE OF *ZYMOMONAS* AND HISTORY OF ISOLATIONS

Ciders and Perries

At the turn of this century the taste of the cider-consuming public changed; they began to demand a clear, sweet cider, instead of a rough

and dry farm-made cider. The manufacturers encountered great difficulties, as sweet ciders easily develop a secondary fermentation, i.e., the "cider sickness" (see review [114]), perhaps due to an infection from fruit (11, 29).

Barker and Hillier (12) were the first to study cider sickness extensively, and they gave a description of the bacterium responsible for the typical aroma and flavor. Outbreaks of the disorder occurred from May until the end of summer. The earliest symptoms are frothing and abundant gas formation. In a few days, the gas pressure causes the bottle to explode. The growing bacteria change the aroma and flavor of the cider and reduce its sweetness. The cider attains a marked turbidity, which clears afterwards, with the formation of a heavy deposit. The susceptibility of cider to sickness depends on such factors as the low acidity of the cider apples, the residual sugar in mature cider, and a high storage temperature.

From the complex microflora of sick cider, Barker and Hillier (12) isolated and purified a bacterial strain (strain A [11]) in 1911. The organisms caused the typical strong aroma and flavor upon reinfection in sterile cider. They were motile rods, single or joined in pairs, 2 by 1 μm, with rounded ends; in old cultures, the cells were frequently longer; involution forms were frequently up to 200 μm long, and their ends may be globular or dumb-bell shaped, with a diameter of 25 μm; spores were absent; they were facultatively anaerobic, showing limited and slow growth on solid media; growth was creamy white and slimy; they vigorously fermented glucose and fructose with formation of ethanol and CO_2; sucrose, maltose, and lactose were not fermented. The authors also stressed that cider sickness is not identical with the wine disorders known as "la pousse" and "la tourne" in France. The discovery of representatives of *Zymomonas* is commonly attributed to Lindner (see below) from pulque in Mexico in 1924, but the real discovery of this genus was made in 1911 by Barker and Hillier. Unfortunately, they did not give a latin taxon name to the new organisms.

Barker (11) purified and studied two new isolates from outbreaks of sickness in 1943 and 1948; they were designated by him as strain B and strain C, respectively. Both strains induced cider sickness. The cells were indistinguishable, 2 by 1 μm, occurring singly or in pairs, with involution forms in older cultures. The cells of strain B arranged themselves specifically in chains; strain C grew in complex, clumpy aggregates. Both strains were extremely motile. Strains B and C produced very little aroma; strain A produced a quite different, intense, and characteristic aroma.

In 1950 Millis isolated 33 strains from ciders and from perries; 27 resembled Barker's strain B and six resembled Barker's strain C (114). The former strains were short rods, 2.5 by 1.5 μm, or diplobacilli, 4 by 1.5 μm; they were pleomorphic and gram negative, without spores or capsules. Actively motile when young, their motility ceased after 3 to 4 days. Motility depended on the medium; it was sometimes absent in apple juice. The latter six strains formed rosettes. The isolates resembling Barker's strain B grew only very faintly aerobically on apple juice-yeast extract agar. Anaerobically, the colonies grew to a diameter of 0.3 mm in 2 days and to 3 to 5 mm in 7 to 8 days. They were butyrous, circular, regular, with an entire edge, convex to umbonate, opaque, and pale grey-buff. After 2 to 3 days on apple juice-yeast extract agar, a characteristic sickly sweet aroma was produced, which faded after a week. In the liquid apple juice medium, a compact creamy deposit was formed. The colonies of the isolates resembling Barker's strain C were more convex and less opaque; after 8 to 10 days, they had a curdled look. They spread and became irregularly circular. They, too, produced the characteristic sickly aroma. In the liquid apple juice medium a granular flocculent deposit was formed. In apple juice-gelatine stabs incubated without anaerobic precautions, growth with gas pockets occurred along the stab; there was no growth at the surface. Peptone could replace yeast extract as a source of nitrogen in growth media, but the growth was less vigorous: colonies on the former substrate reached a diameter of 1 mm; on the latter, they were 2 to 2.5 mm. Millis (114, 115) examined the biochemical characteristics of strain 1 from cider and strain 2 from perry. Both strains formed approximately 1.87 mol of ethanol per mol of glucose, CO_2 and some H_2S, acetaldehyde, and lactic acid. Lactose, raffinose, maltose, sucrose, galactose, rhamnose, arabinose, xylose, dulcitol, mannitol, or sorbitol was not fermented. Catalase was present. The tests for indole, methyl red, acetylmethylcarbinol, nitrite from nitrate, ammonia, and gelatin liquefaction were negative. Strain 1 grew in the pH range 3.75 to 7.9, its optimal pH range being 4.5 to 6.5. The optimal temperature range was from 25 to 31°C. At 41°C, no growth occurred. To inhibit the growth of the cider sickness bacillus, 0.075 to 0.1% SO_2 was necessary. Table 1 compares strains 1 and 2 from Millis (114, 115) with *Zymomonas anaerobia* NCIB 8227 and *Z. mobilis* strains.

Millis (114) artificially induced cider sickness in apple juice and cider. Apple juice infected

TABLE 1. *Comparison of a few Zymomonas strains and their effect on cider*[a]

Names (as used by Millis)	No. and origin of strain[b]	Motility	Fermentation of:			Cell deposit in liquid medium	pH range of growth	Characteristics of cider after infection
			Sucrose	Raffinose	Sorbitol			
Zymomonas anaerobia var. *pomaceae* = cider sickness bacillus	Strain 1 (Millis)	+	−	−	±	Compact	3.5–7.9	Typical aroma and flavor of sickness
	Strain 2 (Millis)	+	−	−	−	Flocculent	3.5–7.9	Typical aroma and flavor of sickness
Zymomonas anaerobia	NCIB 8227	+	−	−	±	Flocculent	3.4–7.5	Putrid yeasty aroma; flavor similar to that of sickness but not typical
Zymomonas mobilis	Strain 1 TH Delft = ATCC 10988	−	+ (Gas)	+	+	Flocculent		Similar to sickness but not identical
	Strain 2 TH Delft	+	+ (Gas)	−	+	Compact		Foul aroma; flavor similar to sickness but not typical
	Strain 3 TH Delft	+	+ (Gas)	−	+	Flocculent		Not characteristic of sickness; foul aroma; flavor unpleasant and harsh

[a] According to Millis (114, 115).
[b] See also Table 5.

with cider sickness bacteria alone fermented more rapidly than when infected with the cider sickness plus the natural microflora. In the latter competition conditions, the cider sickness bacteria remained present in small numbers only. There is some evidence that small numbers of sickness bacteria are present in the fresh juice. This author also studied the development of cider sickness in the presence of cider yeasts. The yeast population declined as the sickness bacterium developed, but rose again after day 45 (Fig. 1). When the cider sickness bacteria alone were inoculated, only slight population fluctuations occurred until day 130; in the next 20 days, the cell count declined rapidly. With a heavy yeast suspension (curve 10:2), the sick aroma and flavor were lost after 130 days. Millis commented: "The observation made by the cider-makers that the aroma and flavour of sickness can be 'boiled' out of cider by encouraging a vigorous yeast fermentation has been supported by these results." Probably a delicate ecological and physiological balance exists between the cider yeast flora and the cider sickness bacteria, both competing for the sugar, the N source, and growth factors.

To prevent the development of cider sickness in ciders, a high acidity of the cider and low storage temperatures were recommended by Barker and Hillier (12). As a possible treatment of the disorder Grove (77) proposed mixing with sharp ciders, addition of tartaric acid, or the addition of brewer's yeast. Barker (11) proposed the pasteurization of cider at 60°C.

Carr (28) stated that the cider disorder known as "framboisé" in France is caused by *Z. anaerobia*. It is questionable whether *Zymomonas* is involved in every case of framboisé.

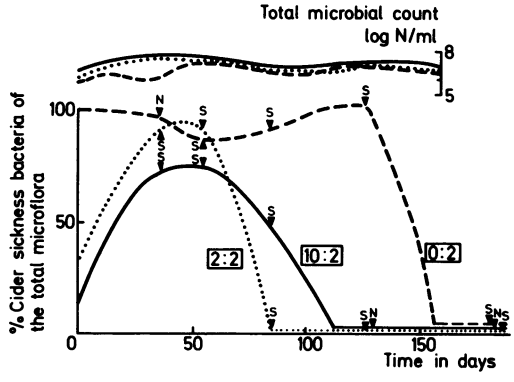


FIG. 1. *Cider sickness bacteria in competition with cider yeasts in cider. A fermented cider was centrifuged and inoculated. All cider samples were inoculated with 2% (by volume) of an actively fermenting culture of cider sickness bacteria. The sample 0:2 received no cider yeast; the samples 2:2 and 10:2 were inoculated with 2 and 10%, respectively, of an actively fermenting cider yeast suspension. The inoculated ciders were stored in a cellar. The ciders were tasted by a panel. Abbreviations: S, Sick ciders; N, normal ciders showing no symptoms of sickness (data from Table 17 of Millis [114]).*

Guittoneau et al. (80) found the fruity odor and taste of "framboisé" ciders to be due to high acetaldehyde concentrations. They attributed the accumulation of this compound to a symbiotic growth of yeasts and acetic acid bacteria. Bidan (24) isolated lactic acid bacteria and acetic acid bacteria from "framboisé" ciders. He stated that these disorders with more than 100 mg of acetaldehyde per liter may be due to various organisms. Auclair (8) pointed to the strict anaerobiosis and low rH values of the

ciders developing the framboisé disorder. Pollard (127) supposed that the "framboisement" of ciders is caused by two different agents, i.e., the anaerobic cider sickness bacillus *Zymomonas* and the aerobic acetic acid bacteria. The means to fight both causative agents of the disorder of "framboisement" are therefore not necessarily the same.

Fermenting *Agave* Sap

During his stay in Mexico in 1923 to 1924, Lindner studied the aguamiel fermentation. Aguamiel is the sugary sap of the *Agave*; it is fermented to obtain pulque, an alcoholic beverage containing approximately 4 to 6% ethanol. Lindner (102) discovered the causal organism of the fermentation, a bacterium which he called *Termobacterium mobile* (now *Zymomonas mobilis* subsp. *mobilis*). This very motile bacterium was able to ferment sucrose, fructose, and glucose to ethanol, CO_2 and some lactic acid. Maltose and lactose were not fermented (104). Lindner (105) suggested that the occurrence of these bacteria is restricted to tropical regions, where they account for the alcoholic fermentations in palm wines, chica beer, etc., and for bread manufacture. They might even have been the causal organisms in the preparation of the "solid beers" of the Arabs and the ancient Babylonians.

Lindner's isolate was studied by Kluyver and Hoppenbrouwers (89) and characterized as follows. The cells occur as diplobacilli, 4 to 5 μm by 1.4 to 2.0 μm; they are motile with polar flagella and gram negative; they have no endospores. After 2 days at 30°C on beer wort agar, the colonies are white, circular, convex, and 1 mm in diameter. Very poor growth occurs on peptone agar or peptone gelatin. There is good growth on beer wort agar with 2% sucrose and in yeast water with glucose, particularly when 2% $CaCO_3$ is added. The pH of the peptone glucose medium should be adjusted to 6.5 with lactic acid or phosphate solutions. The optimum temperature is 30°C. Catalase is present. The organisms are facultative anaerobes, but support a certain degree of aerobiosis in the presence of fermentable sugars. In anaerobiosis 45% of the glucose is transformed to ethanol, and 45% is converted to CO_2, some lactic acid, and traces of acetylmethylcarbinol. Glucose, fructose, and sucrose are fermented; mannose, galactose, maltose, lactose, raffinose, arabinose, dextrine, and mannitol are not. After repeated subculturing in glucose media, a lag phase of 48 h occurs upon reinoculating a sucrose medium. Concentrated glucose solutions of up to 25% were still fermented.

Neither Lindner nor Kluyver and Hoppenbrouwers (89) related the pulque bacteria with the cider sickness organisms described much earlier by Barker and Hillier (see above).

Beer

Shimwell (150, 151) isolated *Zymomonas* for the first time from beer, from the surface of brewery yards, and from the brushes of cask-washing machines. The cells were gram-negative plump rods, 2 to 3 by 1 to 1.5 μm in young cultures and longer in old cultures, without endospores. Motile or nonmotile strains occurred in beer from the same brewery. For the nonmotile strains the name *Saccharomonas anaerobia* var. *immobilis* was proposed. Motility was lost within 3 days. The cells formed rosette-like clusters. The surface colonies, incubated in CO_2 on glucose beer agar, were irregularly circular, 1 mm in diameter, cream colored, sometimes with translucent margin, convex, entire edged, butyrous, and granular. The deep colonies were lenticular. In glucose beer agar, a dense filiform-to-beaded growth occurred along the stab, but no surface growth. In nutrient broth, yeast extract, sugar-free beer, or in any medium without glucose or fructose, there was no growth. In glucose yeast extract broth, the cells grew with a deposit on the wall of the tube and a dense deposit on the bottom. No growth occurred on aerobically incubated agar slopes. The optimum temperature was 30°C. The thermal death point was 60°C for 5 min. The cells grew between pH 3.4 and 7.5. Maltose, sucrose, and lactose were not fermented. In glucose-containing beer, ethanol, CO_2, a little acetaldehyde, and H_2S were produced, but neither acetylmethylcarbinol nor diacetyl. The indole reaction and the nitrate reduction were negative. Hopping had no effect on growth. The culture died off quickly when the fermentation stopped.

Microbial beer spoilage is restricted to a few genera of bacteria and yeasts. According to Ault (9), the most frequently occurring spoilage bacteria in breweries are *Bacillus, Sarcina, Micrococcus, Clostridium, Lactobacillus, Pediococcus, Acetobacter, Gluconobacter,* coliform bacteria, *Flavobacterium,* and *Zymomonas.* Priest et al. (130) pointed to *Citrobacter freundii, Enterobacter aerogenes, Enterobacter cloacae, Hafnia alvei, Klebsiella aerogenes, Hafnia protea, Serratia, Achromobacter, Acinetobacter,* and *Pseudomonas* as wort contaminants.

Recently, *Zymomonas* has been recognized as a serious beer contaminant. In cask or keg beers, *Zymomonas* infections can occur because of anaerobiosis and the presence of priming

sugars. These bacteria produce a heavy turbidity and an unpleasant odor of rotten apples due to traces of acetaldehyde and H_2S (9). In warm weather, spoilage can occur within 2 to 3 days (9, 132). Harrison et al. (81) stated that the features mentioned above make *Zymomonas*, "a particularly important organism to eradicate from high-speed keg beer processing plants." High acetaldehyde concentrations in beers are due to *Zymomonas* (34) (see below).

Zymomonas has not been reported in lager beers. Its presence during the lager fermentation should not be excluded since glucose, sucrose and fructose are present. However, the low temperature of the process, 8 to 12°C, is unfavorable for the development of these bacteria.

The ecology of *Zymomonas* in breweries has been studied by Dadds (33).

Fermenting Palm Sap

Palm wines are alcoholic beverages obtained throughout the tropics from the spontaneous fermentation of the sugary sap of palm trees. The different tree species that are tapped for the preparation of palm wines are given in Table 2. Distillates from palm wines are known in many areas around the world. The chroniclers of the early Euro-African contacts already mentioned palm wine and its preparation (Pigafetta and Lopes in 1591 [125], Capelle in 1641 [27], Jean-François de Rome in 1648 [52], and Zenobio da Firenze in 1820 [37]).

Different modes of tapping the palm sap are practiced: (i) the tree of the oil palm *Elaeis guineensis* Jacq. is felled, and the sap is collected in a shallow notch in the stem or by cutting the terminal bud. (ii) After a preliminary clearing of the tree, the bracts of the male inflorescense are cut off and a hole is made at its base. The hole is left to dry for 2 days and is then reopened to collect the sap in a gourd. The tapping of a tree is continued for 2 to 3 weeks, the hole being reopened twice a day while the collected sap is removed. (iii) Martin (109) described the tapping of *Borassus flabellifer* L. in Cambodia. (iv) In India, Sri Lanka, Indonesia, and the Philippines the male inflorescence of *Cocos nucifera* L. is beaten, and the sap from the wounded flowers is collected. (v) The tree is cleared, and the sap is tapped through a hole under the terminal bud. (vi) The terminal bud of *Raphia* trees is cut, and the sap is tapped from the wound. This method finds application in different parts of Zaïre. A detailed description of the tapping methods used for the

TABLE 2. *Palms from which palm wine are obtained*[a]

Name of palm	Location
Acromia vinifera Oerst	Nicaragua, Panama, Costa-Rica
Arenga pinnata (Wurmb.) Merr. (Syn. *A. saccharifera* Labill.)	Far East
Attalea speciosa Mart.	Brazil, Guyana
Borassus aethiopum Mart.	Tropical Africa
Borassus flabellifer Linn.	India, Cambodia, Java
Caryota urens Linn.	India
Cocos nucifera Linn.	India, Sri Lanka, Africa
Corypha umbraculifera L.	Sri Lanka
Elaeis guineensis Jacq.	Africa
Hyospathe elegans Mart.	Brazil, Guyana
Hyphaenae guineensis Thonn.	West Africa
Jubaea chilensis (Molina) Baillon	Chili
Mauritiella aculeata (H. B. and K.) Burret (Syn. *Lepidococcus aculeatus* H. Wendl and Drude)	Brazil, Venezuela
Morenia montana (Humb. and Bonpl.) Burret (Syn. *Kunthia montana* Humb. and Bonpl.)	Brazil
Nypa fruticans Wurmb.	Sri Lanka, Bay of Bengal, Philippines, Carolines, Salomons Island
Orbignya cohune (Mart.) Dahlgren ex Standley (Syn. *Attalea cohune* Mart.)	Honduras, Mexico, Guatemala
Phoenix dactylifera Linn.	North Africa, Middle East
Phoenix reclinata Jacq. (Syn. *Phoenix spinosa* Schum. and Thonn.)	Central Africa
Phoenix sylvestris (L.) Roxb.	India
Raphia hookeri Mann and Wendl.	Africa
Raphia sudanica A. Chev.	Africa
Raphia vinifera Beauv.	Africa
Scheelea princeps (Mart.) Karsten (Syn. *Attalea princeps* Mart.)	Brazil, Bolivia

[a] See references 3, 25, 30, 84, and 131.

oil palm and *Raphia* palms is given by Tuley (167, 168).

The yield of palm sap from one palm tree may be considerable. Simonart and Laudelout (152) mentioned a felled *Elaeis guineensis* tree that produced 150 liters of sap within 32 days. In Zaïre, one of us found that, from *E. guineensis* Jacq. trees, approximately 3 liters of sap per day was collected during 14 to 21 days; from the *Raphia* trees 2 to 11 liters of sap per day is collected during 3 to 6 weeks (172).

As the sap oozes out in the gourd or in any other container, it is infected by microorganisms colonizing the stalk of the male inflorescense, the tap holes and the surrounding parts covered with hairy outgrowths (57, 58), the tapping equipment, and the gourd itself. The sap from different gourds is pooled, and the palm wine is generally consumed within 24 h. At that moment the palm wine is a whitish, sugary, and acid alcoholic beverage (3, 166). Vanderijst (169) described it very well: "Le vin de palme frais, en pleine fermentation, est un liquide blanchâtre, trouble, pétillant, à saveur sucrée et aigrelette. Il est peu alcoolisé. C'est une boisson rafraîchissante, nourissante, légèrement stimulante et d'un goût assez agréable."

The characteristics of palm wines depend on many factors such as the period of tapping the palm trees, the palm species, the storage period, the season, etc. (1, 2, 60, 61, 123, 166). Palm wine contains 0.1 to 7.1% ethanol, depending on the stage at which it is collected. A normal palm wine contains approximately 4 to 5% ethanol and has a pH of 3 to 4. The alcoholic fermentation of the sap starts in the collection gourds. As the fermentation proceeds, the pH of palm wine drops to 3 to 4, and the presence of tartaric, malic, pyruvic, succinic, lactic, *cis*-aconitic, citric, and acetic acids can be demonstrated (170, 172). The hydrolyzable sugar content of the sap of *Arenga pinnata* (Wurmb.) Merr., *Borassus aethiopum* Mart., *Borassus flabellifer* L., *Cocos nucifera* L., *Caryota urens* L., *Nypa fruticans* Wurmb. ranges from 8 to 16.5% (20, 25, 30, 117, 123, 131, 152, 166, 172). The main constituent is sucrose. The sap also contains small amounts of glucose, fructose, maltose, raffinose, and maltooligosaccharides (13, 60).

The preservation of palm wines has been the object of some reports. Faparusi (56) found that the sodium metabisulfite concentration needed to suppress microbial growth is at least 500 μg/ml. Okafor (122) estimated an average intake of 500 mg of metabisulfite, per day and per person, for a daily consumption of about 600 ml of treated palm wine. Both data show that the acceptable maximal daily intake of 0.35 mg of sulfite per kg of body weight (116) is largely exceeded and not acceptable for human consumption. Faparusi and Bassir (59) demonstrated that the bark of *Abryga gabonensis* Baill. (*Sacoglottis gabonensis* Urb.) possesses some bacteriostatic activity and is able to reduce the rate of souring of palm wine if the bacterial population is not too high. Okafor (123) suggested a pasteurization at 70°C for 30 min combined with sorbic acid as the most useful means of preserving palm wine. A pasteurization unit for the processing of *Raphia* wine with a capacity of 1,000 liters per day has been set up in Cameroon. As the preparation of palm wines is certainly not to be considered as a marginal activity in African countries, one can say that the processing of palm wines opens up considerable technical and commercial prospects (65).

Palm wines have rather complex microfloras (Table 3), but it is certain that *Zymomonas* strains are very important bacterial constituents. They are largely responsible for the alcohol content and for the frothing because of CO_2 formation. CO_2 and small amounts of lactic and acetic acids (see below) contribute to the acidity. The production of some acetaldehyde and the characteristic fruity odor of *Zymomonas* probably positively influence the odor and the taste of palm wine.

Zymomonas strains are extremely well adapted to this ecological niche: sucrose, glucose, fructose, amino acids, and growth factors are present in the palm sap. The organisms are resistant to ethanol and grow at low pH and in anaerobic conditions. Whether *Zymomonas* occurs in all palm wines, whether antagonism or synergism with other organisms occurs, what degree of competition for the sugar there is with yeasts, and at which stage maximum *Zymomonas* density is reached remain unanswered questions. *Zymomonas* has been isolated from palm wines on many occasions. Roelofsen (139) was the first to isolate these bacteria from fermenting *Arenga pinnata* (Wurmb.) Merr. sap in Java (Indonesia). His strain was unfortunately lost and new isolates were made at the request of A. J. Kluyver by G. Derx and C. O. Schaeffer in 1949 from *Arenga* sap, respectively, in Bogor and in Bandung, Java, Indonesia. One of us (J.S.) isolated a number of *Zymomonas* strains from *Elaeis* and *Raphia* palm wines in Western and North-Western Zaïre. These strain will be extensively discussed below. Also, Faparusi (58) and Okafor (122) reported on the isolation of *Zymomonas* from Nigerian *Elaeis* and *Raphia* palm wines, respectively.

TABLE 3. *Microorganisms in palm wines*

Organism	Source
Bacteria	
Acetobacter	56, 59, 60, 122
Acetobacter rancens var. *vini*	152
A. roseus	58
Enterobacter aerogenes	58
Bacillus	60, 122
Brevibacterium	122
Corynebacterium	56
Klebsiella	13, 122
Lactobacillus brevis	58, 122
L. leichmannii	152
L. pastorianus	122
L. plantarum	13, 56, 60
Leuconostoc mesenteroides	13, 56, 58, 60
Micrococcus	13, 58, 60, 122
Pediococcus	60
Sarcina	56, 58
Sarcina lutea	122
S. marcescens	58
Streptococcus	56, 60, 122
Zymomonas	58, 122, 139, 170
Fungi	
Aspergillus	59, 60
Aspergillus flavus	58
A. niger	58
Candida	60, 121
Candida mycoderma	59
C. tropicalis	60
Endomycopsis vini	121
Kloeckera apiculata	121, 152, 170
Mucor mucedo	58
Pichia	13
Pichia pastoris	58
P. membranaefaciens	58
Rhizopus nigricans	58
Rhodotorula	152
Saccharomyces	121
S. cerevisiae	13, 56, 59, 60, 152, 170
S. chevalieri	4, 170
S. ellipsoideus	170
S. exiguus	121
S. florentinus	121
S. markii	121
S. pastorianus	170
S. rosei	121
Saccharomycodes ludwigii	4
Schizosaccharomyces	152
Schizosaccharomyces pombe	4, 13, 60

The repeated isolation of *Saccharomyces*, *Zymomonas*, lactobacilli, and *Acetobacter* tends to confirm the hypothesis of Simonart and Laudelout (152) that palm wine may be the result of mixed alcoholic, acetic, and lactic fermentations. The role of several organisms isolated is not understood and may not be essential. Little investigation has been done on the succession of some microorganisms in palm wine. Okafor (122) counted yeasts, *Streptococcus*, *Lactobacillus*, *Acetobacter*, and *Micrococcus* spp. in three different palm wines at 24 h intervals for several days; he found considerable differences. His conclusions consisted of the following general trends (Fig. 2). (i) The yeasts tended to remain constant in numbers, i.e., 10^6 to 10^9/ml. (ii) *Micrococcus* occurred most consistently in the samples. (iii) Lactic acid bacteria were important components of the microflora. (iv) Certain gram-negative bacteria, e.g., *Serratia* and *Klebsiella* spp., were only present during the first 3 days. (v) *Acetobacter* developed after day 3. Unfortunately, the author did not count *Zymomonas* cells present.

Fermenting Sugarcane Sap

Gonçalves de Lima et al. (70) isolated *Zymomonas* strains from fermented sugarcane juice (caldo-de-cana-picado) from Northeast Brazil. The bacteria were gram-negative rods, 2 to 6 by 1 to 2 μm, small and large cylinders, single or in pairs, rarely forming chains of a few cells. They were very motile with mono- or lophotrichous flagella. The colonies were very typically mucoid. In liquid media, the bacteria developed abundantly with a flocculent deposit. Fermentation still occurred at 42°C. Growth occurred after 15 to 18 h of incubation both in aerobiosis

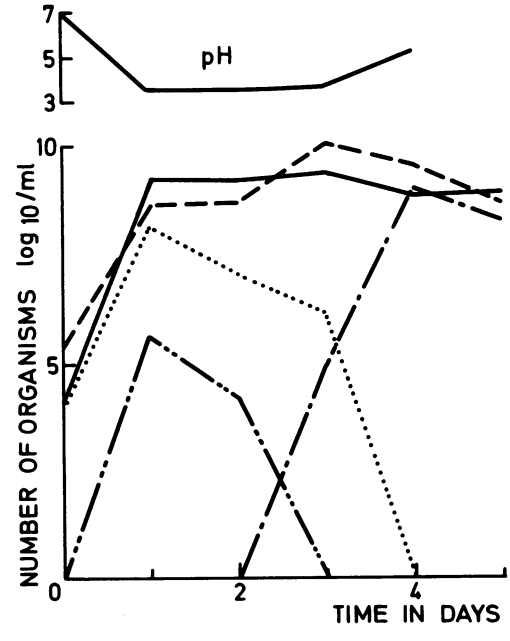


FIG. 2. *Sequence of some microorganisms in palm wine (schematically from Okafor [122]). Symbols:* (———) *yeasts,* (------) *Micrococcus,* (· · · · · ·) *Lactobacillus,* (-· · · · · ·) *Acetobacter, and* (-· · · · · ·) *Enterobacter.*

and in anaerobiosis. Glucose, fructose, and saccharose were fermented with gas production. Lactose, maltose, xylose, raffinose, rhamnose, inositol, arabinose, galactose, mannitol and mannose were not metabolized. Acetylmethylcarbinol, indole, urease, and H_2S were not produced. Nitrate was not reduced. Catalase was present. The antagonistic action against 39 strains of bacteria and fungi has been established. In another publication (72), it was demonstrated that this antagonistic action was not caused by the pH drop per se or by the lactic, succinic, or acetic acids produced by *Z. mobilis*: "The antagonistic activity proved to be a specific activity but at some degree related with the increased acidity produced by the metabolic activity." A new taxon *Z. mobilis* var. *recifensis* was created for these organisms (see below).

Ripening Honey

Ruiz-Argueso and Rodriguez-Navarro (141) have isolated *Z. mobilis* from ripening honey and, occasionally, also from the bee. The two main microbial genera present in ripening honey were *Gluconobacter* and *Lactobacillus*.

Some Technological Applications

The two properties of *Zymomonas*, quick consumption of glucose by an alcoholic fermentation and the lowering of pH of the medium, provoked much interest in the early 1930s among German microbiologists and suggested a number of promising applications for these bacteria. Lindner (104) used them to preserve extracted cuttings of sugar beets. After 80 days of anaerobic incubation, the taste, aroma, and color were good, and the beet flavor had disappeared. The pH fell to 4.4. *Z. mobilis* had no harmful effects on man or cattle. The preservation of the leaves of sugar beets with these bacteria was also tried successfully, and a winy aroma developed (104). Beer draff, treated with *Z. mobilis* for preservation (103), and potatoes treated in the same way (104) were both eagerly accepted by cattle.

Lindner (103, 104) used *Z. mobilis* to ferment diluted molasses and milk enriched with fermentable sugars. He obtained beverages with pleasant flavors.

Schreder (107) used the property of *Z. mobilis* to ferment glucose and fructose, but not maltose, to prepare from beer wort a near beer containing approximately 0.7% ethanol. A malt with low protein content was needed; it was mashed and inoculated with the bacteria. After 3 days, a nutritive beer was ready for consumption. It still contained maltose, dextrin, and peptone and had a characteristic taste and a stable foam. "Das Getränk ist erfrischend und durstanregend, 'smeckt nach mehr' und gibt bei dem Genusz schon das Gefühl, das es nicht müde macht," said Lüers (107). Another beverage of low alcohol content was prepared by Lindner from a malt-coffee-milk mixture. We are not aware that these preparations and beverages became widely appreciated.

In several countries of the Third World, some research is being conducted in order to promote the valorization of the cheap and abundantly occurring sugar contained in fruit and plant saps, and also of the plant starch, in order to prepare alcoholic beverages. We are convinced that *Zymomonas* may be of considerable interest for the development of new beverages and for the industrialization of traditional beverages. Sánchez-Marroquín et al. (142, 143) developed a new technological method for the production of pulque. It is based on the use of selected cultures of *Saccharomyces carbajali* and *Z. mobilis* as alcohol producers, *Lactobacillus* spp. for the lactic acid fermentation, and *Leuconostoc mesenteroides* to obtain the desired degree of viscosity. In an industrial plant, a production of 50,000 liters per day was attained. The beverage obtained was comparable to the pulque prepared traditionally by spontaneous fermentation.

The alcoholic beverages of the world can arbitrarily be subdivided in two groups: (i) those obtained by fermentation of the simple sugars (mono- and disaccharides) present in plant juices or extracts from, e.g., apple, pear, cherry, prune, grape, currant, raspberry, elderberry, strawberry, rhubarb, orange, pineapple, palm, sugarcane, cocoa—usually called wines; and (ii) those obtained by fermentation after saccharification of the starch present in such materials as corn, sorghum, soybean, millet, plantain, rice, barley, wheat—usually called beers. The empirical use of *Zymomonas* in the preparation of palm, *Agave*, and sugarcane wines suggests that these bacteria might be responsible for a large spectrum of alcoholic beverages from a great variety of plant juices in tropical areas around the world. It would be very important to examine a great variety of these beverages for the presence of *Zymomonas*.

Another possible technological application of *Zymomonas* is in the large-scale production of ethanol. All fermentation equations reported in the literature (see below) and our own experience with some 40 strains show that all *Zymomonas* strains produce more than 1.5 mol of ethanol per mol of glucose added. The most suitable strains produce about 1.9 mol of ethanol from each mol of glucose, which is

about as much as *S. cerevisiae*. The conditions must be strictly anaerobic; otherwise, the yield of ethanol is smaller. In aerobic conditions, only about 1 mol of ethanol is produced from 1 mol of glucose. *Zymomonas* grows and ferments very fast. Its preference for low pH prevents foreign contamination. These bacteria still grow in high glucose and ethanol concentrations. Kluyver and Hoppenbrouwers (89) reported up to 10% (wt/vol) ethanol from 25% glucose solutions. Almost half of our strains grew in media with 40% glucose; how much ethanol is produced still remains to be determined. These organisms seem to be very promising agents for the industrial production of ethanol from molasses or other sugary juices. As far as we know, however, this possible application has not yet been pursued.

Therapeutic Use

"Aguamiel" seems to have been used for therapeutic purposes already in the Aztec tradition (Clavigero, cited in [73]). Lindner (101) mentioned the therapeutic use of fresh or concentrated juice of *Agave* in cases of renal and metabolic diseases, and he considered aguamiel and the beverages made with *Zymomonas* as the tropical counterparts of yogurt and kefir.

On several occasions, Lindner (103, 106) recomended the use of *Zymomonas* as a competitor to avoid the disagreeable effects of a putrefactive flora. The harmlessness of *Zymomonas* towards man was demonstrated by Lindner (106): ". . . . habe ich ruhig einen biologischen Selbstmord riskiert, indem ich in Gegenwart meiner Mitarbeiter einen vollen Suppenlöffel einer ausgeschleuderten Masse dieses Tm (*Termobacterium mobile*) verschluckt habe." He reported further on the successful application of *Zymomonas* against foot-and-mouth and Bang's diseases in cattle and against purulent furuncles and wounds in man.

In vitro, the antagonistic effect of *Zymomonas* against a number of bacteria and filamentous fungi has been examined by Gonçalves de Lima et al. (70, 72, 73). There are four reports on the therapeutic applications of *Zymomonas* cultures in cases of chronic enteric and gynecological infections. The data are summarized in Table 4.

History of Individual Strains

Until recently, few *Zymomonas* strains were available. Most of the biochemical and physiological studies have been carried out with *Zymomonas* strains ATCC 10988 and NCIB 8227 (see below). The history of the isolates is summarized in Table 5. Several strains are no longer available. Exact and extensive information on some isolates remains scarce, despite intensive searching.

DETECTION, ISOLATION, AND IDENTIFICATION OF THE GENUS *ZYMOMONAS*

Detection

A suitable detection (32) medium contains (all in percent, weight/volume) malt extract, 0.3; yeast extract, 0.3; glucose, 2; peptone, 0.5; and actidione, 0.002. The pH is adjusted to 4.0. The medium is dispensed in screw-capped bottles (capacity, 25 ml) with Durham tubes, 20 ml/bottle, and sterilized. Ethanol is added to a final concentration of approximately 3% (vol/vol). The presence of *Zymomonas* is indicated by abundant gas production at 25 or 30°C after 2 to 6 days. False-positive results may occur due to lactobacilli or some wild yeasts; a Gram stain is recommended. Positive tests should be completed by the final identification of *Zymomonas* as described in Identification, below. The sensitivity of the method is estimated to be 1 to 5 infection units per ml of inoculum.

A detection method using immunofluorescent staining has a sensitivity of 160 to 2,500 cells per ml (35). Harrison et al. (81) developed a rapid nonspecific method for the detection of low concentrations of viable brewery organisms such as yeasts, *Zymomonas*, *Lactobacillus*, and *Acetobacter* as a quality control for pasteurized or sterilized products. It is based on the change in pH of a medium containing 2% glucose, 0.3% yeast extract (Difco), and 0.01% Tween 80 at pH 7, inoculated with the sample to be tested.

Isolation

Barker and Hillier (12) isolated the cider sickness organisms on beer wort gelatine as minute dotlike colonies after 11 days at 22°C. Shimwell (150) enriched the sample anaerobically in sterile beer with 2% glucose and subsequently plated out anaerobically on glucose beer agar. Barker (11) isolated the cider sickness bacteria on malt extract gelatin plates. An isolation medium with apple juice has been used by Millis (114, 115): apple juice was treated overnight with pectozyme and filtered, and then 1% yeast extract (Difco) and 0.001% actidione were added. The pH was adjusted to 4.5. A 10-ml sample of the infected cider was centrifuged, and the deposit was streaked on plates with the above medium. The plates were incubated anaerobically in the McIntosh and Fildes jar at 25°C.

One of us (162) described the isolation of *Zymomonas* from fresh Zairese palm wines in

TABLE 4. *Therapeutic uses of Zymomonas*

Disorder treated	No. of cases		Treatment	Strain used	Source
	Treated	Cured			
Chronic enterocolitis Chronic cystitis	6 1	6 1	Liquid cultures, 300 ml a day between the meals	Ag-11	De Paula Gomes (51)
Vaginitis caused by *Candida albicans*, *Trichomonas vaginalis*, and *Neisseria gonorrhoeae*	20	20	Tampons impregnated with *Zymomonas*, changed daily	Ag-11	Wanick et al. (177)
Gynecological infections by *Candida albicans*, *T. vaginalis*, and *N. gonorrhoeae*	40	40	Liquid cultures and agar jellies	IA-CP1	Wanick and Cavalcanti Da Silva (176)
Colpitis and vulvovaginitis caused by *E. coli*, *Haemophilus vaginalis*, *N. gonorrhoeae*, *T. vaginalis*, and *Staphylococcus* spp.	95	82	Tampons	IA-CP1	De Souza and De Souza (53)

WL differential medium (Difco). Three methods can be used: (i) different dilutions of the sample are mixed with the WL differential medium and poured in thick layers in petri dishes before solidifying; (ii) samples are plated and covered by a top layer of medium; (iii) the samples are streaked on the medium in petri dishes and incubated in the GasPak anaerobic system. The last procedure seems most convenient to us. In the WL differential medium the colonies are lenticular, 1 to 4 mm after 4 to 5 days at 30°C, and deep green. For the isolation of *Zymomonas* from palm wines it is very important to use a young wine, about 24 h old, as we have never been able to isolate these bacteria from palm wines over 48 h old. We have not found *Zymomonas* in fermenting sugarcane juice or in fermenting cocoa in Zaïre, where we expected these bacteria to occur.

Richards and Corbey (137) proposed a two-step isolation procedure. (i) The deposit from 25 ml of centrifuged spoiled beer was enriched at 30°C in beer at pH 4.0 with 4% glucose and 0.3% yeast extract. (ii) Cultures were streaked on the same solidified medium and incubated anaerobically at 30°C.

Identification

The following features permit the identification of *Zymomonas* (i) rods 2 to 6 μm long with an unusual cell width of 1 to 1.4 μm, (ii) gram negative, (iii) no spores, (iv) if motile, by 1 to 4 lophotrichous flagella, (v) no growth on nutrient agar or in nutrient broth, (vi) anaerobic, but tolerates some oxygen, (vii) fermentation of glucose and of (viii) fructose to (ix) nearly equimolar amounts of ethanol and CO_2, (x) oxidase negative, (xi) 47.5 to 49.5% of guanine plus cytosine (G+C). These 11 features may be considered as the minimal description of the genus *Zymomonas*. It should be noted that the common generic description of *Zymomonas* (88, 90, 151) comprises fewer features.

The key for the identification of pseudomonads proposed by Hendrie and Shewan (82) has to be improved with respect to the identification of *Zymomonas*. They characterized it as (i) gram-negative rods, (ii) motile with polar flagella, and (iii) growing anaerobically. Motility is not an essential feature for *Zymomonas*, since only 30% of the strains were motile (see Cell morphology). On the other hand, the almost quantitative fermentation of glucose to ethanol and CO_2 should be included as a basic feature.

In a tentative key for the identification of spoilage bacteria in brewing, Ault (9) characterized *Zymomonas* as (i) gram-negative organisms, (ii) forming no acid from ethanol, and (iii) fermenting glucose to ethanol.

Some Commonly Used Media and Growth Conditions

Standard media. The liquid standard medium we use most often contains 0.5% yeast extract (Difco) and 2% glucose, 9 ml per test tube. For the solid standard medium, 2% agar is added. Kluyver and Hoppenbrouwers (89) recommended the addition of 2% $CaCO_3$ to this medium; however, it is not necessary. *Zymomonas* cultures should be transferred every 2 to 3 weeks.

Apple juice medium. This contains four-times-diluted apple juice, 1% yeast extract (Difco), and 10% gelatin, pH 5.5, in screw-capped bottles. In this medium the cider sickness bacteria remain viable for 17 weeks (115).

Beer media. Shimwell used media contain-

TABLE 5. *Index of Zymomonas strains*[a]

Original strain designation or name of strain as received by us	Strain no.	Isolation			Strain reported to be dead (−) or strain in our collection (+)	Isolated by (references)
		Source	Place	Yr (or publication date)		
Cider sickness bacillus	A	Sick cider	Bristol, U.K.	1911	−	Barker and Hillier (12)
Zymomonas mobilis	ATCC 10988 = NCIB 8938 = NRRL B-806 = Queensland 410 = L192 Delft = 1TH Delft = DSM 424 = IMG 1655	Fermenting *Agave* juice	Mexico	1924	+	Lindner (102–104)
Z. mobilis	Queensland 410 ex NCIB 8938 ex ATCC 10988	Fermenting *Agave* juice	Mexico	1924	+	Lindner (102–104)
Z. mobilis		Fermenting sap of *Arenga pinnata*	Java, Indonesia	1935	−	Roelofsen (139)
Z. anaerobia		Bad beer	U.K.	(1937)		Shimwell (150)
Cider sickness bacillus	B	Sick cider	Bristol, U.K.	1943		Barker (11)
Cider sickness bacillus	C	Sick cider	Bristol, U.K.	1948		Barker (11)
Z. mobilis subsp. *pomaceae*	NCIB 11200 = ATCC 29192 = I (Barker); very likely either B (Barker) or 1 (Millis)	Sick cider	Bristol, U.K.	Before 1951	+	Deposited by Barker with A. J. Kluyver, Delft, in 1951
Z. mobilis	3TH Delft	Fermenting sap of *Arenga*	Bogor, Java, Indonesia	1949	+	Derx (164)
Z. mobilis	6TH Delft	Fresh sap of *Arenga* (tuak)	Bandung, Java, Indonesia	1949	+	Schaeffer (164)
Z. anaerobia	NCIB 8227	"Bad" beer	U.K.	Before 1951	+	Almost certainly in the Research Department, H. J. Bunker, Barclay Perkins & Co. Ltd., London
Z. anaerobia	Queensland 409 ex NCIB 8227	"Bad" beer	U.K.	Before 1951	+	
Z. anaerobia subsp. *pomaceae*	1	Sick cider	Bristol, U.K.	1950		Millis (114)
Z. anaerobia subsp. *pomaceae*	2	Sick perry	Bristol, U.K.	1950		Millis (114)
Z. mobilis	AG 11	Fermenting *Agave* juice	Mexico	1950	+	Gonçalves de Lima et al. (71)
Z. mobilis	IA-113	Subclone of AG 11	Mexico	1950		Gonçalves de Lima et al. (71)
Z. anaerobia var. *pomaceae*	NCIB 8777		Bristol, U.K.	Before 1956		Deposited by Carr in 1956
Z. anaerobia	D-364 (Sneath)	Beer		(1966)	−	Shimwell (155)
Z. mobilis	Z6 = NCIB 11199 = ATCC 29191	Fermenting *Elaeis* sap	Kinshasa, Zaïre	(1967)	+	In the Department of Microbiology, Lovanium Univ., Kinshasa, Zaïre
Z. mobilis	Z1, Z2, Z3, Z5, Z7, Z8	Fermenting *Elaeis* sap	Kinshasa, Zaïre	(1967)	+	
Z. mobilis	VP1, VP2, VP3, VP4	Fermenting *Elaeis* sap	Kinshasa, Zaïre	1969	+	Swings (162)
Z. mobilis	7.4	Fermenting *Elaeis* sap	Tshela, Zaïre	1969	+	Swings (162)
Z. mobilis	70.1, 70.2, 70.3, 70.7, 70.8, 70.9	Fermenting *Elaeis* sap	Kinshasa, Zaïre	1970	+	Swings (162)
Z. mobilis	70.10, 70.11, 70.12, 70.14	Fermenting *Elaeis* sap	Kinshasa, Zaïre	1970	+	Swings (162)
Z. mobilis var. *recifensis*	CP1, CP2, CP3	Fermenting sugarcane juice	Recife, Brazil	(1970)	+ +	Gonçalves de Lima et al. (70)

TABLE 5—*Continued*

Z. mobilis var. *recifensis*	CP4	Recife, Brazil		+	Gonçalves, de Lima
Z. mobilis	5.1, 5.3, 5.4, 5.5	Fermenting *Raphia* sap	Bwamanda, Zaire	1971	+ + Swings (162)
Z. mobilis	17.1, 17.2, 17.3, 17.4	Fermenting *Raphia* sap	Mobayi, Zaire	1971	+ + Swings (162)
Z. mobilis	42.1, 42.2, 42.3, 42.4	Fermenting *Raphia* sap	Bwamanda, Zaire	1971	+ + Swings (162)
Z. mobilis	23.1, 23.3	Fermenting *Raphia* sap	Bwamanda, Zaire	1971	+ + Swings (162)
Z. anaerobia	NCIB 10565 = S30.2 (Carr and Passmore)	Apple pulp	U.K.	1970	+ Passmore (29)
Z. anaerobia	1	Beer	U.K.	(1972)	+ + Dadds (33)
Z. anaerobia	2	Beer	U.K.	(1972)	+ + Dadds (33)
Z. anaerobia	2.1	Subclone of 2		1972	+ + Swings
	2.2	Subclone of 2		1972	+ + Swings
Z. mobilis	B70	Infected British ale	License premises, U.K.	(1973)	+ Dadds et al. (36)
Z. mobilis		Primed beer	Sheffield, U.K.	(1974)	– Richards and Corbey (137)
Z. mobilis		Fermenting *Elaeis* sap, tapholes	Nigeria	(1974)	Faparusi (58)
Zymomonas		Fermenting *Raphia* sap	Nigeria	(1975)	– Okafor (122)
Z. mobilis		Ripening honey, bee	Madrid, Spain	(1975)	Ruiz-Argueso and Rodriguez-Navarro (141)

[a] The new nomenclature of the strains listed is given in Table 7 and in the text (Classification and Nomenclature). When the exact year of isolation is not known, the year of publication is given in parentheses. The strains are listed in chronological order.

ing beer with 2% glucose. He recommended weekly transfers. For maintenance, he preferred a glucose-beer gelatin stab culture. A beer medium made of a primed ale with 2% glucose and 0.1% yeast extract has been used by Dadds et al. (36) for the detection of volatile sulfur compounds. Several commercial beers unfortunately contain disinfectants. Their use in media cannot be recommended.

MYPG medium. This medium contains 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% glucose, and 2% agar (179).

Synthetic media. The minimal synthetic medium of Kluyver and Hoppenbrouwers (89) contains 2% glucose, 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$ in tap water.

A synthetic medium for the determination of amino acid and growth factor requirements was described by Belaïch and Senez (17). Van Pee et al. (174) added the following vitamins: *p*-aminobenzoic acid, biotin, folic acid, cyanocobalamin, lipoic acid, nicotinic acid, pyridoxine, thiamine, riboflavin (1 μg each per ml), as well as guanine, adenine, hypoxanthine, cytosine, and uracil (60 μg each per ml).

The medium of Gibbs and DeMoss (67). It contains 1% glucose, 1% tryptone, 1% yeast extract (Difco), 0.5% KH_2PO_4 in 98 ml, plus 2 ml of a salt solution with 0.8% $MgSO_4 \cdot 7H_2O$, 0.16% $MnSO_4 \cdot 4H_2O$, 0.04% NaCl, and 0.04% $FeSO_4 \cdot 7H_2O$.

Zymomonas can be grown at 25 to 30°C. Most authors prefer to incubate *Zymomonas* anaerobically, but this is only necessary for surface growth on slants or petri dishes.

TAXONOMY OF *ZYMOMONAS*

In the present section we shall critically review several proposals made in the literature on the nomenclature and classification of this genus. A good modern taxonomy of a bacterial taxon requires a numerical analysis of many and diverse phenotypic data, the DNA base composition (percent G+C), the degree of genome DNA relatedness (percent D, DNA "homology") determined by DNA-DNA hybridizations, a comparison of protein electropherograms, etc. We shall summarize and discuss the data that led us (50) to propose an improved taxonomy of this genus.

Numerical Analysis of the Phenotype

The original description of *Z. mobilis* by Kluyver and Hoppenbrouwers (89) and of *Z. anaerobia* by Shimwell (151) comprised some 35 phenotypic features. Millis used some 40 features in comparing six *Zymomonas* strains (see

below, Classification and Nomenclature). Numercial analysis of phenotypical information requires a larger number of strains and of tests; therefore we (50) used 38 *Zymomonas* strains from diverse origins (Zaïrese fermenting palm sap, Mexican pulque, British spoiled beer, and sick cider). We determined 138 phenotypic features for these strains. The extensive description of these features will be given in Phenotypical Description, below. We shall limit ourselves here to their numerical analysis. The main new features we added were: several C sources for growth, antibiotic sensitivity, growth factor requirements, stimulation of growth by amino acids, and the susceptibility to dyes and some other compounds. We calculated the simple matching coefficients S_{SM} (156). These values were clustered both by single linkage (154) and by unweighted average linkage (156). The dendrogram has been published (50).

Twenty-eight features tested are present in every strain. These features thus constitute a valuable description of *Zymomonas* and considerably enlarge the minimal description given in the section, Identification. They can be summarized as follows. *Zymomonas* cells are gram-negative rods, 2 to 6 μm long, 1 to 1.4 μm wide, usually occurring in pairs. If motile, they have one to four polar flagella; motility may be lost spontaneously. Colonies in standard medium (see above) are glistening, white, or cream colored with a diameter of 1 to 2 mm after 2 days at 30°C. The colony edge is regular. A fruity odor of variable intensity, according to the strain, was observed. They grow on and ferment glucose and fructose, mainly to almost 2 mol of ethanol and 2 mol of CO_2 (gas) per mol of hexose fermented. The ratio, moles of ethanol produced to moles of glucose fermented, is at least 1.5. Some lactic acid and traces of acetylmethylcarbinol are formed. All strains can grow in a medium with 2% yeast extract and 20% glucose. The optimal pH for growth is 7.3. The final pH after 3 days at 30°C in standard medium is 4.8 to 5.2. The cells can grow in standard medium in the presence of 5% ethanol. Catalase is present. The cells require pantothenate and biotin for growth. They can grow in standard medium in the presence of 0.1% neutral red. They reduce 2,3,5-triphenyltetrazolium chloride and the dyes methylene blue and thionin. Their growth is sensitive to either 500 μg of sulfafurazole, 30 μg of novobiocine, 10 μg of tetracycline, or 10 μg of fusidic acid on disks.

All *Zymomonas* strains tested by us lack the following 74 features. They form no spores or capsules; they have no detectable lipids in the cells. They do not grow in 0.5% yeast extract broth, in 1% peptone broth, in liquid standard medium with 2% NaCl, in liquid standard medium at pH 3.05, or, after three transfers, in the synthetic medium of Kluyver and Hoppenbrouwers. Aerobically, no or poor growth was obtained on standard medium agar. They do not grow on nutrient agar. They do not grow on or ferment starch, dextrin, raffinose, D-trehalose, maltose, lactose, D-cellobiose, D-galactose, D-mannose, L-sorbose, salicin, L-rhamnose, D- and L-arabinose, D-xylose, D-ribose, D-sorbitol, dulcitol, D-mannitol, adonitol, erythritol, glycerol, ethanol, Na D-galacturonate, Na DL-malate, Na succinate, Na pyruvate, Na DL-lactate, Na tartrate, or Na citrate. Their growth is not stimulated by L-cysteine, L-glutamate, glycine, L-histidine, L-lysine, DL-methionine, L-ornithine, DL-serine, L-threonine, or L-tyrosine. For growth, they require neither lipoic acid, folic acid, nicotinic acid, nor *p*-aminobenzoic acid. They are oxidase negative. They do not form indole. They reduce neither nitrate, neutral red, nor safranin. They hydrolyze neither gelatin nor Tween 60 or 80. They are not sensitive to 0.01% actidione, 5 U of bacitracin, 10 μg of gentamycin, 10 μg of kanamycin, 10 μg of lincomycin, 30 μg of nalidixic acid, 10 μg of neomycin, 5 U of penicillin, 300 U of polymyxin, 10 μg of streptomycin, or to the vibriostatic compound 0/129.

Thirty-seven features occur in some strains but not in others. They are (percentage of strains with features present are given in parentheses): motility (26), formation of rosettes (50), pleomorphism (63), the nature of the cell deposit (compact [65], granular to flocculent [34]), the growth on and the fermentation of sucrose (50), the formation of H_2S (11), the presence of the decarboxylases for L-ornithine (11), L-arginine (82), and L-lysine (47), the presence of urease (24), growth in standard medium with 0.5% NaCl (97) or 1% NaCl (71), growth in standard medium at pH 3.5 (45) or 7.55 (84), growth in standard medium at 34°C (97) or at 38°C (74), growth in 2% yeast extract with 40% glucose (55), a pH <4.8 after growth in standard medium at 34°C for 7 days (2), growth after a second transfer in the synthetic medium of Kluyver and Hoppenbrouwers (11), growth decrease by omission of one or more of the following amino acids: L-alanine (8), L-arginine (11), L-asparagine (5), L-hydroxyproline (11), L-isoleucine (5), L-leucine (21), DL-phenylalanine (2), DL-proline (5), DL-tryptophan (2), or L-valine (47), the requirement of either riboflavin (8), thiamine (2) or cyanocobalamin (8), the resistance to 10 μg of ampicillin (39), 10 μg of

cephaloridine (29), 30 μg of chloramphenicol (2), 10 μg of erythromycin (89), 10 μg of methicillin (97), or 30 μg of vancomycin (53).

The phenotypic similarity between all the strains is very high: they all form one cluster above 88% S_{SM}. The strain Z6 from Zaïrese palm wine was calculated to be the phenotypic centrotype, the most representative strain of *Z. mobilis* subsp. *mobilis*. It was deposited as NCIB 11199 and ATCC 29191. Within this subspecies, the fermentation of sucrose and the motility are not correlated with other features. To maintain the separate species *anaerobia* and its variety *immobilis* is not justified (see below).

Three strains in our collection are slightly aberrant. One was isolated in Bristol from sick cider. We deposited it as *Z. mobilis* subsp. *pomaceae* ATCC 29192 and NCIB 11200. This strain forms the border of the group, with an average difference of 17 features and six correlated features, differentiating this strain from all the other zymomonads (Table 6). Two other *Zymomonas* strains, NCIB 8777 and NCIB 10565, have been investigated less extensively, but they are almost identical to ATCC 29192.

DNA Base Composition and DNA Genome Size

We determined the DNA base composition of 41 *Zymomonas* strains by thermal denaturation (163). All these strains have a midpoint of thermal denaturation (T_m) between 88.9 and 89.7°C, with an average T_m of 89.3 ± 0.2°C (standard deviation). DNA base composition varies within the narrow range of 48.5 ± 1.0% G+C. The cider sickness organism *Z. mobilis* subsp. *pomaceae* ATCC 29192 is near the bottom of the group, with 47.7% G+C.

A few other percent G+C values have been published; on the whole, they fit well within the range we established. Dadds et al. (36) reported 48.3% for *Zymomonas* NCIB 8938, Kiehn and Pacha (87) found 48.6%, presumably for the same strain, and we found 49.1 and 48.6% for two subcultures. For *Zymomonas* NCIB 8227, we found 49.0% G+C; Dadds et al. (36) reported 47.6% G+C, slightly too low. All the above percent G+C values are close and support the idea that there is great similarity within this genus.

Dadds et al. (36) reported 46.4% G+C for *Zymomonas* NCIB 10565 and 45.1% G+C for *Zymomonas* B70. In our experience, the latter strain is genotypically and phenotypically indistinguishable from all the other *Z. mobilis* subsp. *mobilis* strains. The low percent G+C values of the above two strains strongly suggest that they should be redetermined.

The genome size of some 40 *Zymomonas* strains was determined with the initial renaturation rate method (68, 69). The DNA genome size, expressed as molecular weight, of *Zymomonas* strain 5.3 is 1.53 (± 0.19) × 10^9, and those of the other *Zymomonas* strains are not significantly different. This genome size is rather small, about 56% of the *Escherichia coli* genome. It can accommodate some 1,500 cistrons.

Genome-DNA Relatedness

We determined the degree of genome-DNA relatedness (percent D; DNA "homology") (163) with the initial renaturation rate method (48) in stringent conditions, i.e., those that allow duplex formation to occur only between very closely related or identical polynucleotide sequences.

All strains except one were found to be genetically very similar. The degree of DNA relatedness in the main group is at least 76% D. This main group consists of two subgroups of strains that are almost identical. One subgroup con-

TABLE 6. *Differential features of the cider sickness organism Z. mobilis subsp. pomaceae ATCC 29192 (= NCIB 11200)*[a]

Features	*Z. mobilis* subspecies	
	mobilis	*pomaceae*
Growth in standard medium + 0.5% NaCl	Growth after 1–3 days	No growth
Growth in standard medium at 36°C	Growth after 1 day	Scant growth after 2 days
Growth in standard medium + 0.0075% KCN	Growth after 1–5 days	No growth
Final pH after 7 days of growth at 34°C in standard medium	4.9–5.4	4.7
No. of amino acids stimulating growth	0–5 (exact number depends on the individual strain)	6
Tolerance towards O_2	Various degrees of microaerophily	Most anaerobic strain

[a] Modified from reference 50.

sists of 28 Zaïrese palm wine strains Z1, Z2, Z3, Z5, Z6, Z7, Z8, 7.4, 17.1, 17.3, 17.4, VP1, VP2, VP3, 42.1 42.2, 42.3, 42.4, 70.1, 70.2, 70.3, 70.7, 70.9, 70.11, 70.14, 5.1, 5.4 and 5.5. Their average DNA relatedness is 94% D, with a minimum of 88%. The second subgroup consists of the Zaïrese palm wine strains 17.2, 70.10, 70.12, strains 2.1, 2.2, 409, and NCIB 8227 from infected British beer, and strains 410 and ATCC 10988 from Mexican pulque. Their average percent D is 96. Both subgroups are linked at an average of 82% D. Both subgroups do not represent taxonomic units, since they have no characteristic phenotypic features, protein electropherograms, or percent G+C.

There are no genetic differences between named *Z. mobilis* and *Z. anaerobia* strains, between sucrose-fermenting and sucrose nonfermenting strains, or between motile and nonmotile strains.

The pomaceae organism ATCC 29192 has a distinctly different genome: it has less than 32% D with all subspecies *mobilis* strains.

Similarity of Protein Electropherograms

Kersters and De Ley (86) pointed out that the cells of each bacterial strain, grown in standardized conditions, should always produce the same set of protein molecules, within experimental error. The protein gel electropherograms, prepared in rigorously reproducible conditions, were indeed found to be specific for each strain. Kersters and De Ley (86) developed techniques for preparing these electropherograms and comparing them by computer-assisted techniques.

We applied these techniques to our battery of strains; the dendrogram of quantitative correlations has been published (164). A visual comparison of the stained gels of the *Zymononas* strains reveals a very similar pattern, composed of 15 bands (Fig. 3). The three strains of *Z. mobilis* subsp. *pomaceae* (NCIB 10565, NCIB 8777, ATCC 29192) have a different protein pattern, which makes them easily recognizable and stresses again their separate taxonomic position. Strain identification is possible upon visual inspection in three other cases, i.e., the strains 7.4 and 70.7, both lacking band H, and strain *Z. anaerobia* 1, showing a very strong P band. However, all our *Zymomonas* strains, except three, have a very similar, and sometimes identical, soluble-protein composition, whether the strains were isolated from a variety of Zaïrese palm wines, Mexican fermenting *Agave* juice, Indonesian *Arenga* sap, or British deteriorated beers. The highly correlated protein patterns of the strains reflect the close genetic resemblance shown by DNA-DNA relatedness, percent G+C, and numerical analysis of the phenotype. The strain Z6 = ATCC 29191 is the electrophoretic centroid strain, i.e., the most representative strain of the cluster. We found no noticeable differences of the protein patterns between strains named mobilis and anaerobia, between sucrose-fermenting and sucrose-nonfermenting strains, and between motile and nonmotile strains.

Infrared Spectra of Intact Cells

In the past, several authors have tried to use the infrared (IR) spectra of intact bacterial cells as an aid in differentiation and identification (7, 26, 76, 92, 94, 120, 128, 138, 148).

The broad, diffuse absorption bands in the IR spectra of intact *Zymomonas* cells (Fig. 4) are due to components common to all bacterial cells, i.e., lipids, proteins, and polysaccharides. The IR spectra of various genera, species, and types are therefore very similar. This clearly reduces the usefulness of IR spectra for bacterial classification.

Reproducible differences, which are not always correlated with other classifications, occur, e.g., in *Acetobacter* and *Gluconobacter* (148), lactobacilli (75), *Desulfovibrio* and *Desulfotomaculum* (26), and Actinomycetales (7, 92, 94, 128). Minute, but reproducible, differences occur in the shape and intensity of the absorption regions of *Zymomonas* spectra from 800 to 1,000 cm^{-1} and from 1,000 to 1,200 cm^{-1}. Strains Z2, 70.7, and VP2 have a typical uneven absorption maximum between 1,000 and 1,200 cm^{-1}. *Z. mobilis* subsp. *pomaceae* ATCC 29192 has a shoulder at 960 cm^{-1}, whereas all the other strains have a distinct peak (162).

Thus, the IR spectra of *Zymomonas* strains are too similar to allow taxonomic conclusions. In some strains, specific IR details occur.

Serology

There seem to be at least two serological groups in *Zymomonas* (36; P. A. Martin, personal communication). The following strains belong in serotype 1: NCIB 8227, NCIB 8938, B72, Z2, and 42.1. In serotype 2 belong strains NCIB 10565, B70, and 42.1. In his Ph.D. thesis, Dadds (33; no permission for quoting data was obtained) lists a few more strains. By comparison with our own phenotypic data we found no correlation between the serotypes and other features of all these strains. The serological differences seem to be of no validity for species separation in *Zymomonas*, but could have some use in strain identification.

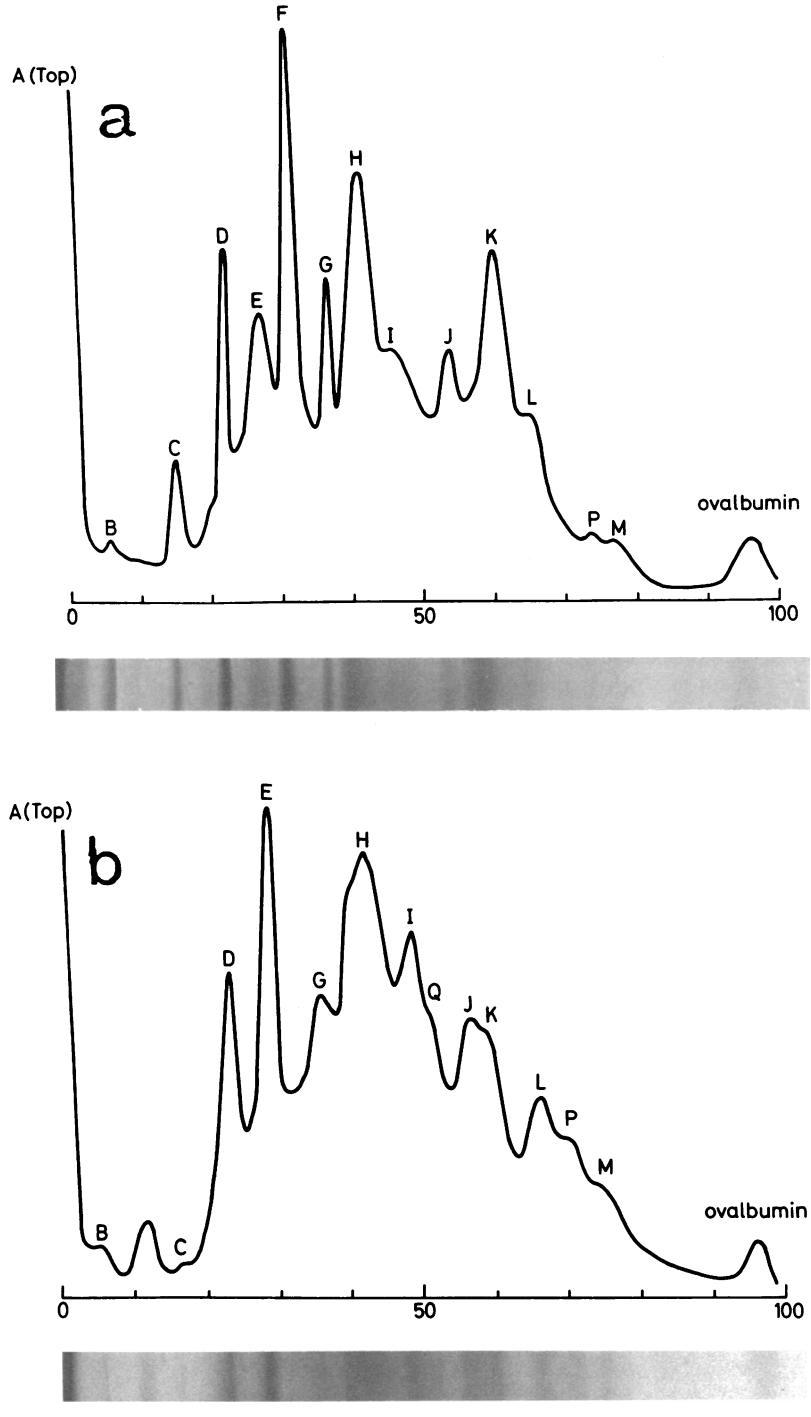


FIG. 3. *Protein electropherograms and densitometer scannings of Zymomonas ATCC 29191 (a) and ATCC 29192 (b).*

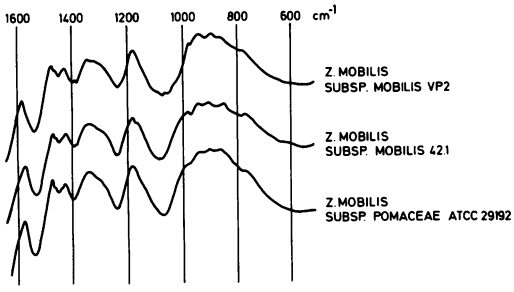


FIG. 4. *IR spectra of Zymomonas.*

Classification and Nomenclature

Since these bacteria were first named in 1928, they have been moved into five different genera, but, since 1936, they have been apparently securely fixed in *Zymomonas*. They have received some 20 different names, which are listed in Table 7.

One name requires immediate comment. Shimwell (150) named his new strain *Achromobacter anaerobium*. In a footnote he suggested also a new name *Saccharobacter* as suitable, "if . . . it should be considered that the characteristics of this organism justify its inclusion in a new genus." However, the name *Saccharobacter* has no nomenclatural value, since it is in contradiction with Principle 8 of the International Code of Nomenclature of Bacteria (96) and it is not validly published, according to Rule 28b.

Barker and Hillier (12) were the first to describe the cider sickness organism, but they did not give it a latin name. It was the real discovery of the genus *Zymomonas*. Lindner described in 1905 (100) a variety of fermentative bacteria that deteriorated beer wort; he assigned them to *Termobacterium*, an ill-defined genus comprising diverse small nonsporing rods, as *T. fuscescens, T. album,* etc. It is therefore not surprising that he assigned the fermentative pulque bacteria to the same genus as *T. mobile* (102). *Termobacterium* is no longer accepted as a genus.

Kluyver and Hoppenbrouwers (89) renamed Lindner's isolate *Pseudomonas lindneri* according to the scheme of Lehmann and Neumann (98). The genus *Zymomonas* was created by Kluyver and van Niel (90), "for polarly flagellated bacteria causing alcoholic fermentation." They designated *Z. mobilis* (Lindner) as the type species.

Shimwell (150) isolated a strain from beer causing dense turbidity with unpleasant odor and flavor. Although the organism was presented as a powerful and dangerous cause of a beer disease, the nature and the economic importance of this disorder remained obscure and undetailed. The organism was described. Its anaerobic nature, approaching a microaerophile, was stressed by the new species name *Achromobacter anaerobium*. The same author proposed, in 1950 (151), a new genus *Saccharomonas*, "to indicate the physiological resemblance to *Saccharomyces* and the morphological kinship with *Pseudomonas*" for bacteria producing a quantitative alcoholic fermentation of glucose. Two species were proposed:

Saccharomonas anaerobia Shimwell
Saccharomonas anaerobia var. *immobilis* subsp. nova
Saccharomonas lindneri (Kluyver and Hoppenbrouwers) Shimwell.

S. anaerobia was advanced as the type species. Its inability to ferment sucrose (see below) was stressed in italics; the organism was described as, "anaerobic but microaeroduric (not microaerophilic)." Shimwell made no experimental comparison between both species *anaerobia* and *lindneri*; for the latter, he extracted literature data from *Bergey's Manual*, 6th ed., from Kluyver and Hoppenbrouwers (89), and from Schreder et al. (144–147). Therefore, the great similarity between both presumed species did not become apparent. Shimwell (151) deserves credit for relating *Pseudomonas lindneri, Achromobacter anaerobium*, and Barker and Hillier's cider sickness bacteria. However, the name *Zymomonas* has clear precedence over the name *Saccharomonas* Shimwell 1950, the latter being illegitimate [Rule 51b (2), International Code (96)].

Kluyver (88) introduced the genus name *Zymomonas* in *Bergey's Manual*, 7th ed. He proposed two species:

Z. mobilis (Lindner 1928) Kluyver and van Niel 1936
Z. anaerobia (Shimwell 1937) Kluyver 1957.

Barker and Hillier's cider sickness organisms were mentioned but remained unnamed. Kluyver mentioned an unpublished comparative study of cultures of *Z. mobilis*, *Z. anaerobia*, and the cider sickness organism, carried out in 1951, showing that all these organisms are closely related, but data were not presented.

Millis (115) was the first to give the scientific name *Zymomonas anaerobia* var. *pomaceae* to the perry and cider sickness organisms. Millis compared two strains, 1 and 2, (isolated by her from ciders and perries) with *Z. anaerobia* NCIB 8227 (from "bad" beer, United Kingdom) and with three strains of *Z. mobilis* (Lindner's

TABLE 7. *List of names given to representatives of the present genus Zymomonas*

Zymomonas mobilis subsp. *mobilis* (Lindner) De Ley and Swings, 1976
Synonyms
Termobacterium mobile Lindner 1928, 253
Pseudomonas lindneri Kluyver and Hoppenbrouwers 1931, 259
Saccharomonas lindneri (Kluyver and Hoppenbrouwers) Shimwell 1950, 182
Zymomonas mobile (sic) (Lindner) Kluyver and van Niel 1936, 399
Zymomonas mobilis (Lindner) Kluyver and van Niel 1936, 399
Zymomonas mobilis var. *anaerobia* Richards and Corbey 1974, 243
Zymomonas congolensis Van Pee and Swings 1971, 311
Achromobacter anaerobium Shimwell 1937, 509
Saccharobacter Shimwell 1937, 509
Saccharomonas anaerobia Shimwell (Shimwell) 1950, 181
Zymomonas anaerobia Shimwell (Kluyver) 1957, 199
Zymomonas anaerobia var. *anaerobia* (Shimwell) Carr 1974, 353
Saccharomonas anaerobia var. *immobilis* Shimwell 1950, 182
Zymomonas anaerobia var *immobilis* (Shimwell) Carr 1974, 353
Zymomonas mobilis var. *recifensis* Gonçalves de Lima, De Araújo, Schumacher and Cavalcanti Da Silva, 1970, 3
Zymomonas mobilis subsp. *pomaceae* (Millis) De Ley and Swings, 1976
Synonyms
Cider sickness organism Barker and Hillier 1912, 78
Zymomonas anaerobia var. *pomaceae* Millis 1956, 527

original pulque organism; Derx's strain isolated from *Arenga* sap in Bogor, Java; Schaeffer's strain isolated from *Arenga* sap in Bandung, Java) (see also Table 1). These six organisms were identical in 20 features. The cider sickness organisms resembled *Z. anaerobia* NCIB 8227 and differed from the *Z. mobilis* strains in two features only (lack of sucrose fermentation; no gas from sorbitol). The main difference between *Z. anaerobia* NCIB 8227 and the cider sickness organisms resided in the changes in aroma and flavor produced in cider.

In *Bergey's Manual*, 8th ed. (28), *Zymomonas* is divided into:

Z. mobilis (Lindner) Kluyver and van Niel
Z. anaerobia (Shimwell) Kluyver
Z. anaerobia var. *anaerobia* (Shimwell) Carr
Z. anaerobia var. *immobilis* (Shimwell) Carr
Z. anaerobia var. *pomaceae* Millis.

This classification showed that no progress had been made in the taxonomy of *Zymomonas* since 1956. It introduced some unfortunate errors: (i) both Kluyver (88, 89) and Shimwell (151) knew that *Z. mobilis* strains can lose and regain the ability to metabolize sucrose; this important feature was not mentioned in *Bergey's Manual*, 8th ed. (ii) The "cider sickness organism" is *Z. anaerobia* var. *pomaceae* (Millis) and not *Z. anaerobia* (Shimwell) Kluyver. (iii) There is no proof that *Z. anaerobia* is the cause of the unpleasant flavor of the "framboisé" in France.

Our extensive reexamination of many strains of *Zymomonas* with modern methods (50, 163, 164) shows that a more realistic classification of this genus is now possible. We summarize our arguments herewith.

(i) The main reason to maintain two species, *mobilis* and *anaerobia*, was the supposed difference in the ability to ferment sucrose (28). This conclusion was based on work with a few strains only. However, this difference is illusive. From the observations of Kluyver and Hoppenbrouwers (89), Dadds et al. (36), and Richards and Corbey (137), it follows that the ability to ferment sucrose is an inducible phenomenon. We found no correlation whatsoever between growth on, and fermentation of, sucrose with other features of *Zymomonas* (see above). The enzymic difference between sucrose-fermenting and sucrose-nonfermenting strains is probably one or at most two enzymes (levansucrase [EC 2.4.1.10] and perhaps invertase [EC 3.2.1.26]). It is not justifiable to maintain a separate species status because of such a small difference.

In the past, some authors have already stressed the resemblance between *Z. mobilis* and *Z. anaerobia*. Kluyver (88) wrote that *Z. mobilis* and *Z. anaerobia* are closely related organisms. De Ley (47) stated that *Z. anaerobia* was probably only a variety of *Z. mobilis*. Richards and Corbey (137) came to the same conclusion and proposed, "that the genus *Zymomonas* be represented by one species only, *viz. Z. mobilis* with the second species relegated to variety level as *Z. mobilis* var. *anaerobia*." Van Pee and Swings (171) thought that the existence of both species designations was not justified by morphological and biochemical dif-

ferences. Dadds et al. (36) studied five *Zymomonas* strains and concluded that their work, "casts grave doubts on the necessity for maintaining two species within the genus *Zymomonas*."

(ii) Shimwell (151) introduced the name *Saccharomonas anaerobia* var. *immobilis*. The differences between *Z. anaerobia* and its variety *immobilis* are really minor: the latter variety displays (i) no flagella and no motility, (ii) no rosette-like clusters, and (iii) a slightly different colony shape; physiologically both taxa are otherwise identical. This proposal was continued by Carr (28) as *Z. anaerobia* var. *immobilis* (Shimwell) Carr. However, motility is not a stable feature and may be readily lost. We established that motility is randomly distributed among the strains of *Zymomonas* and that motile and nonmotile strains do not cluster in separate groups (see above). We conclude that a separate taxon for nonmotile strains cannot be maintained.

(iii) The name *Z. congolensis* was proposed by Van Pee and Swings (170). However, it was published as a nomen nudum, without type strain. We established (50) that these strains are indistinguishable from our other *Z. mobilis* strains and that a separate species status is not justified.

(iv) Gonçalves de Lima et al. (70) studied strains isolated from caldo-de-cana-picado (fermenting sugarcane juice) and named them *Z. mobilis* var. *recifensis*. They are characterized by very mucoid colonies, a high growth yield in liquid medium at 25 to 42°C, the capacity to ferment at 42°C, a great tolerance towards oxygen, the absence of H_2S formation, and a typical antagonistic spectrum against a number of bacteria and fungi. This taxon was not mentioned in *Bergey's Manual*, 8th ed. (28). We recently examined two strains labeled *Z. mobilis* var. *recifensis* CP3 and CP4 (kindly supplied by O. Gonçalves de Lima) by phenotypical and protein electrophoretic techniques. Both are almost identical with the strains of the subspecies *mobilis*. We propose to regard the variety *recifensis* as a synonym of the subspecies *mobilis* (Table 7). Full details will be published elsewhere.

(v) From the evidence we submitted in the sections, Numerical Analysis of the Phenotype, DNA Base Composition and DNA Genome Size, Genome-DNA Relatedness, and Similarity of Protein Electropherograms (50, 163, 164), it follows that nearly all existing strains of *Zymomonas* are so similar, in spite of their greatly diverse origin, that they deserve to be united in one taxon, *Z. mobilis* subsp. *mobilis* (50).

(vi) Three *Zymomonas* strains of our collection were distinctly different. Strain ATCC 29192 was isolated in Bristol from sick cider. It was sent in 1951 by B. T. P. Barker as *Saccharomonas pomaceae* strain I to the collection of the Laboratory of Microbiology, Delft, the Netherlands, where it was renamed *Z. anaerobia* subsp. *pomaceae*. We believe that it is very likely either strain B from Barker or strain 1 from Millis (115). This strain has a low DNA hybridization with all the other strains (163), its protein electropherogram is quite separate (164), and it differs in six phenotypic features from all the other strains in our collection. Strains NCIB 8777 and NCIB 10565 are almost identical with ATCC 29192. The separate nature of these organisms requires its recognition in a separate taxon, *Z. mobilis* subsp. *pomaceae* (50). Some other strains isolated by Barker and Hillier (12), Barker (11), and by Millis (114) from sick cider possibly belong in the same taxon.

(vii) We formally proposed the following new classification (50).

Genus: *Zymomonas* Kluyver and van Niel 1936, 399

Type species: *Zymomonas mobilis* (Lindner) Kluyver and van Niel 1936, 399

1. *Zymomonas mobilis* subsp. *mobilis* (Kluyver and van Niel) De Ley and Swings, 1976
   Lectotype strain: ATCC 10988 = NCIB 8938 = NNRL B-806 = DSM 424 = IMG 1655 = L192 (Lab. Microbiol., Techn. Univ. Delft) = strain 1 (ibid.) = Ampoule no. 410 (Dept. Microbiol., Fac. Med., Univ. Queensland, Australia)
   Phenotypic centrotype: strain Z6 = ATCC 29191 = NCIB 11199
2. *Zymomonas mobilis* subsp. *pomaceae* (Millis) De Ley and Swings, 1976
   Lectotype strain: ATCC 29192 = NCIB 11200 = strain I (Barker).

The synonyms of both subspecies are listed in Table 7.

Relationship Between *Zymomonas* and Other Genera

Opinions on the possible relationship between *Zymomonas* and other genera have varied considerably. When Kluyver and van Niel created the genus *Zymononas* in 1936, they placed it together with 14 other genera such as *Pseudomonas, Acetobacter, Rhizobium,* etc., in the tribe *Pseudomonadeae* of the family *Pseudomonadaceae*. The genus *Zymomonas* was not recognized by R. S. Breed in *Bergey's Manual,* 6th ed., in 1948. The organism from

pulque was still referred to as *Pseudomonas lindneri* Kluyver and Hoppenbrouwers. The genus *Zymomonas* was finally accepted in *Bergey's Manual*, 7th ed. (1957), and described by A. J. Kluyver. Together with the genera *Pseudomonas, Acetobacter, Xanthomonas, Photobacterium* etc., it constituted the family *Pseudomonadaceae*. In *Bergey's Manual*, 8th ed. (28), *Zymomonas* has been moved, as a genus of uncertain affiliation, to Part 8, together with the *Enterobacteriaceae* and the *Vibrionaceae*, the gram-negative facultatively anaerobic rods. The physiological properties (facultative anaerobiosis, fermentation of glucose to ethanol and CO_2, percent G+C) were considered to be as important as the morphological ones (polarly flagellated rods). However, Staley and Colwell (157) found no reassociation between DNA from *Vibrio* and *Zymomonas*. *Zymomonas* is not mentioned in Krassilnikov's "Diagnostik" (91). He mentions that the species *P. mobilis* and *B. lindneri* are unrelated to *Zymomonas*. Prévot et al. (129) accommodate *Zymomonas*, as a genus incertae sedis, in the family of the *Sphaerophoraceae*. This diversity of proposals shows that the exact taxonomic position of the genus *Zymomonas* was obscure.

In our laboratory, the similarities between the ribosomal ribonucleic acid (rRNA) cistrons from several hundred species of gram-negative, heterotrophic, rodlike bacteria were examined (J. De Ley et al., to be published). It was found that there is a good correlation between the degree of rRNA similarity and the overall phenotypic similarity of bacterial genera and/or subgenera. *Zymomonas* belongs in a constellation of genera, represented in Fig. 5. A common feature to most of them is the presence of the Entner-Doudoroff pathway (85). The rRNA cistrons of *Zymomonas, Acetobacter, Gluconobacter, Agrobacterium, Rhizobium, Phyllobacterium*, and a few other genera (on which one of us will report later) have much in common. It is very striking that all these genera have related ecological niches: in, on, or around, plants. We suggest that all these genera are of a common evolutionary origin and may later be united by taxonomists into one family.

Phenotypically, *Zymomonas* resembles most closely the acetic acid bacteria; perhaps they are more like *Gluconobacter* more than *Acetobacter*, because of the polar flagellation, the incomplete Krebs cycle, the occurrence of the Entner-Doudoroff mechanism (which is more widespread in *Gluconobacter* than in *Acetobacter*), and the ready consumption of glucose. *Zymomonas* and acetic acid bacteria both occur on plants and prefer to grow in niches rich in plant juices. They complement each other, since *Zymomonas* produces ethanol, which the acetic acid bacteria in turn, oxidize. In fermenting tropical plant juices, beer, etc., the acetic acid bacteria not infrequently occur simultaneously with or subsequent to *Zymomonas*.

Zymomonas tolerates low pH; many strains grow at pH 3.5; most of them grow at pH 4. Acetic acid bacteria are likewise able to grow at pH 4 to 4.5. This pronounced tolerance to low pH is not common in the bacterial world.

Many *Zymomonas* strains are ethanol tolerant. At 7.7 and 10% ethanol concentrations, 73 and 47%, respectively, of our strains are still able to grow. Many acetic acid bacteria likewise grow readily in 10 to 13% ethanol.

Tolerance of high glucose concentration is another common feature. All our *Zymomonas* strains and most acetic acid bacteria are able to grow on media with 20% glucose. About half of our *Zymomonas* collection is able to grow on media with 40% glucose. Many acetic acid bacteria grow readily in media with 50% glucose.

The outstanding feature of all the acetic acid bacteria is their ability to oxidize ethanol to acetic acid. It is interesting to point out that *Zymomonas* NCIB 8938 is likewise able to oxidize ethanol to acetic acid (17, 38).

Zymomonas assimilates but a small part of its glucose substrate as cell material (17): 98% is fermented and about 2% is used as carbon source. The acetic acid bacteria are likewise inefficient: 85% of glucose is oxidized to gluconate and only 15% is consumed by way of the shunt (49).

There are strong indications of a correlation between the taxonomic grouping of organisms and the regulatory properties of their citrate synthase (178). Gram-negative strictly aerobic bacteria, such as *Pseudomonas*, have a large-molecular-weight citrate synthase, inhibited by reduced nicotinamide adenine dinucleotide (NADH) and reactivated by adenosine 5'-monophosphate (AMP). Gram-negative fermentative bacteria, such as *E. coli*, have large-molecular-weight citrate synthase inhibited by NADH but not reactivated by AMP. Gram-positive bacteria have a small-molecular-weight citrate synthase that is not inhibited by NADH. *Zymomonas* NCIB 8938 and some *Acetobacter* strains have a large-molecular-weight citrate synthase, as expected from gram-negative bacteria; however, the enzyme is not inhibited by NADH (D. Jones, personal communication).

The DNA genome sizes of the 40-odd *Zymomonas* strains which we determined are rather small; the molecular weight is about 1.5×10^9. The DNA genome sizes of the acetic acid bacteria are more diversified: a number of them are

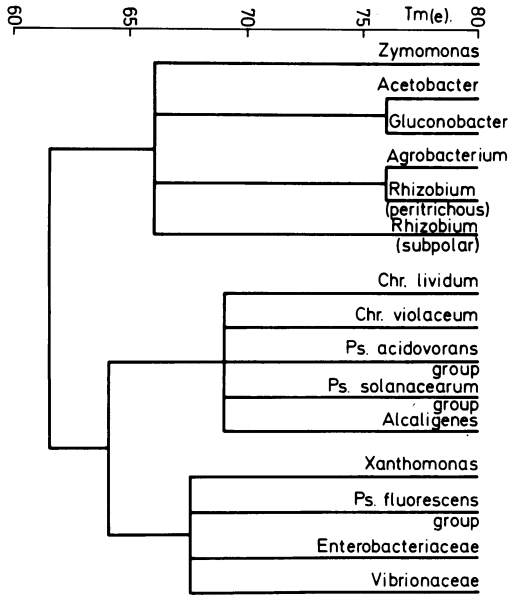


FIG. 5. *Similarities of rRNA cistrons between several taxa. The results are expressed as the thermal stability of the hybrid (melting point of the hybrid in degrees centigrade) (unpublished results of J. De Ley, M. Gillis, J. De Smedt, P. De Vos, P. Segers, and R. Tytgat).*

as small as the *Zymomonas* genome: *A. pasteurianus* subsp. *pasteurianus* 23 k1$^+$ (1.81×10^9) (68), *G. oxydans* subsp. *suboxydans* NCIB 7069 (1.6×10^9), *G. oxydans* subsp. *suboxydans* NCIB 6723, (1.5×10^9), *G. oxydans* subsp. *suboxydans* NCIB 9119 (1.9×10^9); other strains, like *A. aceti* subsp. *aceti* NCIB 8627 and *A. aceti* subsp. *liquefaciens* NCIB 9136, have a genome size of the same order as *E. coli* (2.55×10^9) (all molecular weights from M. Gillis and J. De Ley, unpublished).

PHENOTYPICAL DESCRIPTION: MORPHOLOGY, GROWTH, PHYSIOLOGY AND BIOCHEMISTRY

In this section, we shall summarize and discuss the present knowledge on the phenotype of *Zymomonas*, including both our own data and the information available in the literature.

The Cell

Cell morphology. *Zymomonas* are gram-negative rods, 2 to 6 μm long and 1 to 1.5 μm wide, occurring singly but mostly in pairs. The large cell-width of *Zymomonas* is very striking upon microscopic observation; most other bacteria are only 0.5 to 0.75 μm wide. Most of the strains (70% of our collection) are nonmotile, a minority of some 30% is motile with 1 to 4 polar flagella. Motility is lost in some strains. Forty-five percent of our strains form rosettes, 33% form chains, and 62% have filamentous cells; the latter are sometimes over 28 μm long. These three features are quite characteristic for some individual strains. The strains 17.1 and 17.3 are the only ones with U-shaped cells. No spores, capsules, intracellular lipids, or glycogen have been found.

Macromorphology. In solid standard medium, deep colonies are lenticular, regular, entire edged, butyrous, white or cream colored, and 1 to 2 mm in diameter after 2 days. Anaerobic surface colonies are spreading, entire edged, convex or umbonate, and 3 to 4 mm in diameter. Strain *Zymomonas* 6 TH Delft has an irregular colony edge.

Cellular composition. The main components of the *Zymomonas* cell are given in Table 8. A comparison with some other bacteria is desirable. The dry weight of many bacteria contains 50 to 90% protein. The RNA content of some gram-negative bacteria (e.g., *E. coli*) is about 20% of the dry weight. The adenosine 5'-triphosphate (ATP) pool of 1 to 5 μg/ml in *Zymomonas* during the log phase is similar to that of other bacteria, shown to have 1 to 7.1 μg of ATP per mg (dry weight) (83). *Zymomonas* cells are thus not abnormal in those respects. The DNA content of the bacterial cell depends on the molecular weight of the genome, its state of replication, and the number of nucleoids present. The amount of 2.7% in *Zymomonas* is also in the normal range (about 3.3% in *E. coli*).

According to different authors, 12 to 30% of the dry weight of many bacteria consists of carbohydrates. There is very little reserve mate-

TABLE 8. *Cellular constituents of Zymomonas*[a]

Component	Content (dry wt)
Proteins	
Growth phase	65–69%
Stationary phase	54%
RNA	17–22%
DNA	2.7%
Carbohydrates	4–5%
Poly-β-hydroxybutyrate	0%
Polyphosphate	0%
Sulfur	0.5%
Ammonia	0.1–0.5 μmol/mg
Amino acids	0.02–0.2 μmol/mg
ATP	
Exponential phase	1–5 μg/mg
Starvation	0–0.4 μg/mg

[a] Most data are from Dawes and Large (41); other sources include: sulfur content, from Anderson and Howard (5); and ATP pools, Lazdunski and Belaich (97). The data on ATP pools concern strain NCIB 8938; all other data concern strain NCIB 8227.

rial present in *Zymomonas*. The amount of polysaccharides is low, if any. Polyphosphates (volutin) and poly-β-hydroxybutyrate, which are common in many bacteria, are reported to be absent in *Zymomonas*. However, we observed metachromatic granules in four strains, i.e., Z1, Z6, 7.4, and ATCC 10988. If the amino acids (Table 8) represent the free pool in the cytoplasm, then it can be calculated that each cell contains between 8 and 80 million molecules of them.

Resting cells and starvation. Resting cells are cells that are not growing and dividing. They are usually suspended in buffer, without nutrients for growth, and are frequently starved for a few hours up to a few days. The technique of resting cell preparations is widely used in bacterial physiology and biochemistry. Some properties of resting and starving *Zymomonas* were studied by Dawes and collaborators (38–43).

When *Zymomonas* strain NCIB 8227 is starved, it dies off very quickly. During day 1 (41), about 90% of the cells die. $MgCl_2$ protects, in its presence, about 60% of the cells, initially, from death. From then on the cells continue to die off slowly. During the death and starvation period, most polymeric cell components hardly change. For 118 h, under N_2, the carbohydrate content remains constant at about 4%, and DNA at 2.7%, whereas the protein content falls from about 67% to about 57%. The concentration of ammonia (0.1 to 1 μmol/mg [dry weight]) and of amino acids (0.02 to 0.2 μmol/mg [dry weight]) does not change inside the cell in starvation periods of up to 2 days. Nevertheless, these compounds leak out in the medium. In both strains NCIB 8227 and NCIB 8938, RNA is the only constituent that is significantly broken down in starvation; its content drops drastically to 3 and 5%, respectively. Degradation of RNA is accompanied by release into the medium of material absorbing at 260 nm. This material accounts for all the RNA degraded. The degradation of RNA is suppressed in the presence of 33 mM $MgCl_2$. The RNA is lost more rapidly from strain mobilis NCIB 8938 than from strain anaerobia NCIB 8227. From this isolated case, however, one may not conclude that differences in RNA breakdown constitute a significant species differentiation; they are only strain differences. Upon starvation, the ATP content falls rapidly and levels off to a small value. Cells starved for up to 7 days still contain the enzyme system for producing ATP from glucose and ethanol (40, 41). There seems to exist no correlation between the intracellular ATP concentrations and the viability of the cells.

Growth Response to Different Conditions

Growth in some ordinary media. *Zymomonas* needs a fermentable sugar in its medium for growth, i.e., glucose or fructose, or (for some strains) sucrose. In the liquid standard medium (see Some Commonly Used Media and Growth Conditions), good growth and gas is seen after 24 h. In peptone broth plus 2% glucose, in beer plus 2% glucose, in nutrient broth or nutrient agar plus 2% glucose or in palm juice, good growth occurs. From the strains of our collection, 67% form a compact, and 33% form a slimy-flocculent to granular deposit in liquid media. The type of deposit is strain specific. *Zymomonas* does not grow in 0.5% yeast extract, beer, beer with 0.5% yeast extract, peptone broth, peptone broth with 0.5% yeast extract, nutrient broth or agar, or nutrient agar with 0.5% yeast extract.

In apple juice liquid medium (see Some Commonly Used Media), two cider sickness strains (115) grew with a dense turbidity within 2 to 3 days; afterwards, the medium became clear and a compact (strain 1) or flocculent (strain 2) deposit was formed, darkening with age.

Growth in the liquid synthetic medium of Kluyver and Hoppenbrouwers. All our strains tested were transferred from the standard medium with 1 drop of a Pasteur pipette. Thirty-eight strains develop within 3 days on this synthetic medium (see Some Commonly Used Media and [89]). On a second transfer, only *Zymomonas* strains NCIB 8227, 409, ATCC 10988, and 410 develop. On a third transfer no strain grows. *Zymomonas* is thus not able to grow on this simple synthetic medium, demonstrating the need for a source of organic nitrogen or some growth factors or both. Growth in the first and second transfer is due to carry-over. This synthetic medium has been used in fermentation studies by Kluyver and Hoppenbrouwers (89).

Growth at different pH values. Shimwell (150) found that his *Z. anaerobia* strain grew within the pH range 3.4 to 7.5. Millis (115) determined that two *Zymomonas* isolates from sick cider grew in the pH range 3.5 to 7.9; in the pH range 2.5 to 3.4, there was no growth; at pH 3.5 and 3.6, growth occurred sometimes. The results of the growth tests at different pH values with our collection are summarized in Table 9. The wide pH range for growth, from 3.5 to 7.5, and the acid tolerance are both quite typical for *Zymomonas*. This is not surprising, since in nature they live in acid palm wines and ciders below pH 4. These facts disagree with the assertion of Masschelein (110) that *Z. anaerobia* is acid intolerant and does not develop be-

low pH 4.2.

Growth at different temperatures. The results from the strains of our collection are given in Table 10. Above 30°C, some strains do not grow, and growth at 40°C is rarely observed. Strains from the subspecies *pomaceae* are particularly sensitive, as they do not grow above 34°C. The best test we can recommend to distinguish between both subspecies *mobilis* and *pomaceae* is growth at 36°C.

The cider sickness organisms of Millis (114) did not show any sign of fermentation when incubated for 28 days in the refrigerator; at cellar temperatures (14 to 18°C), the onset of fermentation occurred after 4 days, and, at room temperatures (16 to 26°C), fermentation began after 2 days. The same author (114) found the optimum temperature range to be 25 to 31°C. Dadds et al. (36) noticed a slow growth at 15°C for the strains *Zymomonas* B70 and NCIB 10565. According to Gonçalves de Lima et al. (70), *Z. mobilis* var. *recifensis* strains CP1, CP2, and CP3 grew well between 25 and 42°C. However, we found that strains CP3 and CP4 still grew at 38°C but not at 40°C.

Thermal death point. *Z. anaerobia* is killed by exposure at 60°C for 5 min (150). We tested strains Z1 to Z8 from our collection; they were also killed in these conditions. Grove (78) found that the cider sickness bacillus was killed after 5 min at 55°C.

Growth in the presence of ethanol. It can be expected that *Zymomonas* is alcohol tolerant, as it is isolated from alcoholic beverages containing from 2 to 10% ethanol. A concentration of 5.5% ethanol in liquid standard medium is quite harmless to *Zymomonas*, since all strains of our collection grow. At 7.7 and 10% ethanol, respectively, 73 and 47% of our strains still develop. Growth of the cider sickness bacillus was prevented by 6.5% ethanol (79).

TABLE 9. *Growth of Zymomonas strains in liquid standard medium at different pH values*[a]

Initial pH	% of strains growing
3.05	0
3.5	43
3.7	71
3.85	90
5–7	100
7.50	87
8.0	0

[a] Our data.

TABLE 10. *Growth of Zymomonas strains in liquid standard medium at different temperatures*[a]

Incubation temp (°C)	% of strains growing
30	100
34	97
36	97
38	74
40	5

[a] Our data.

Growth in high glucose concentrations. Kluyver and Hoppenbrouwers (89) observed that the pulque strain was able to grow in and ferment media with 25% glucose.

We tested several tyndallized liquid media containing 2% yeast extract (Difco) and various concentrations of glucose. With 20% glucose, all of our strains grow within 34 h; with 30% glucose, 88% of our strains develop after 2 to 5 days (strains Z1 to Z8 develop within 1 day); with 40% glucose, 54% of our strains grow after lag phases of 4 to 20 days.

Growth in the presence of KCN. We used liquid standard medium with 0.0075% KCN. The strains ATCC 29192, NCIB 8777, NCIB 10565, CP3, CP4, and Ag 11 do not grow. *Zymomonas* strains ATCC 10988, 410, NCIB 8227, and 409 grow after 5 days; 12 other strains are not inhibited by this KCN concentration and develop after 1 to 2 days.

Growth in the presence of NaCl. In liquid standard medium with 0.5% NaCl, only two strains of our collection, i.e., *Z. mobilis* var. *pomaceae* ATCC 29192 and NCIB 8777, do not grow. Seventy-one percent of our strains grow in the presence of 1% NaCl. None of our strains grow in the presence of 2% NaCl. Grove (79) found that the cider sickness bacillus was inhibited by 0.7% NaCl.

Growth in the presence of 0.01% Acti-dione. This antibiotic is a component of the WL differential medium (Difco) we used for the isolation of *Zymomonas*. Millis (115) added Acti-dione (cycloheximide) to the apple juice isolation medium. Dadds (32) included it in a differential medium for *Zymomonas* (see Detection). Fungi are sensitive to 0.01% Acti-dione, and bacteria are not. All our strains tested develop within 4 days.

Growth in the presence of oxgall. Of 17 strains tested, only *Zymomonas* ATCC 10988 and its subculture 410 develop in 0.2% oxgall in solid medium. At 1% oxgall, both strains are inhibited. Much higher concentrations of oxgall are tolerated by *Zymomonas* in liquid media; in 0.2% oxgall, all our strains grow except the pomaceae organism ATCC 29192; in 1% oxgall, 53% of our strains develop, and in 1.5% oxgall, 41% of our strains grow after 2 to 9 days.

Growth in the presence of 0.1% 2,3,5-triphenyltetrazolium chloride (TTC). In both liquid and solid standard media all the strains of our collection grow and reduce TTC to the red formazan.

Growth in the presence of 0.01% thallium acetate. In solid standard medium, growth did not occur.

Growth in the presence of 0.001% cadmium sulfate. Sixty-three percent of the strains grow.

Growth in the presence of the vibriostatic agent 0/129. The importance of this test for the identification of gram-negative, polarly flagellated, and oxidase-positive bacteria (10) prompted us to check it. All *Zymomonas* strains in our collection are insensitive to compound 0/129 (2,4-diamino-6,7-diisopropylpteridine) in solid standard medium.

Tolerance to SO_2. This feature is of practical importance, since SO_2 is the only disinfectant permitted in English cider. Two strains of the cider sickness organism (115) grew at 0.05%, but not at 0.075%, sulfur dioxide. *Zymomonas* is not inhibited at the legally permitted (United Kingdom) 0.02% SO_2 concentration in cider.

Growth in the presence of dyes. Fung and Miller (64) examined the effect of 42 dyes in solid media on the growth of 30 bacterial strains. They showed that gram-negative organisms exhibited greater resistance to dyes than gram-positive strains. Our results show that *Zymomonas* is more sensitive towards dyes than the gram-negative bacteria tested by these authors.

In Fig. 6, the results are summarized of the growth responses of *Zymomonas* in the liquid standard medium with different dyes. Growth inhibition of some strains by certain dye concentrations depended on whether solid or liquid media were used (173). Strain VP2 is remarkably resistant toward dyes. On the whole, our strains are very sensitive to brilliant green.

Malachite green was used by Millis (114) at 0.025% in the isolation medium. With this dye, we found that, even at 0.0005%, some *Zymomonas* strains do not develop; thus, the use of malachite green in isolation media for these bacteria cannot be recommended.

Reduction of dyes and $HgCl_2$. These compounds were incorporated at 0.001% in the liquid standard medium. All our strains reduce Nile blue, cresyl violet, thionine, methylene blue, TTC, and $HgCl_2$. Indigo carmine, neutral red, safranine, and Janus green are not reduced.

Growth in the presence of antibiotics. The tests were performed on solid standard medium using Difco or Oxoid antibiotic disks. Figure 7 represents the sensitivity-resistance pattern of 38 *Zymomonas* strains toward 20 antibiotics. This figure speaks for itself and needs little additional comment.

Greatest sensitivity was shown by strain 70.7, which is inhibited by 11 antibiotics. Strain VP2, mentioned above for its greater resistance against dyes (see Growth in the presence of dyes), has a high resistance against antibiotics: it is inhibited by five antibiotics only. Cross-resistance was observed for the pairs ampicillin-penicillin, gentamycin-kanamycin, gentamycin-streptomycin, and gentamycin-neomycin. The cross-resistance was not always reversible for all the strains with lincomycin-erythromycin.

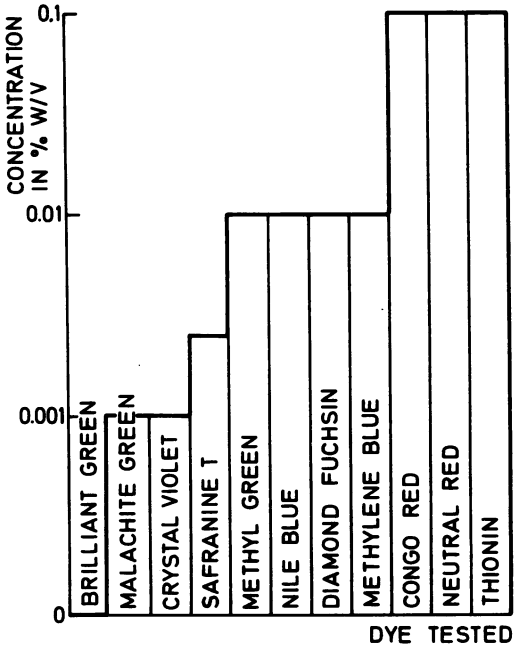


FIG. 6. *Sensitivity of Zymomonas to dyes. The bar heights indicate the dye concentrations at which all of the strains tested grew in the liquid standard medium (data from [173]).*

Carbohydrate Metabolism

Metabolism of glucose and fructose. The almost quantitative fermentation of both hexoses to ethanol and carbon dioxide is a distinctive characteristic of the genus *Zymomonas*. After 12 to 24 h, the liquid standard medium becomes densely turbid, with abundant gas formation.

In 40 *Zymomonas* strains of our collection, the ethanol produced amounts to 1.5 to 1.9 mol per mol of glucose. The original pH in the liquid standard medium is 6.1. The final pH after 3 days at 30°C is 4.8 to 5.2. The acidification is more pronounced upon incubation at 38°C; in several cultures, the pH drops to 4.0. At 34°C, the pH of *Z. mobilis* var. *pomaceae* ATCC 29192 drops to 4.7; with all strains of the variety *mobilis*, the pH drops to 4.9 to 5.4.

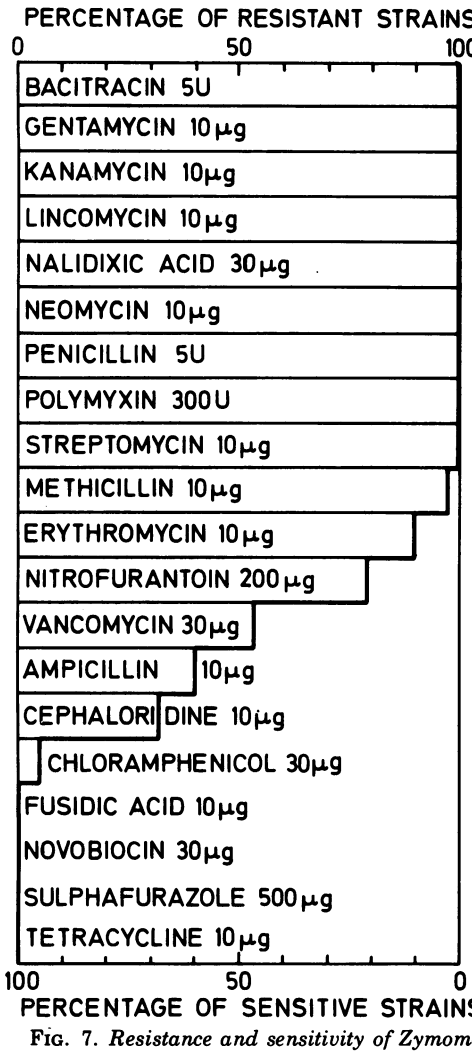


FIG. 7. *Resistance and sensitivity of Zymomonas toward antibiotics.*

In 1912, Barker and Hillier (12) were the first to report on the fermentation by a *Zymomonas* organism (a strain from sick cider). Their isolate fermented glucose or fructose, but not sucrose, maltose, or lactose. They reported: "The gas given off during the fermentation of dextrose consists almost entirely of carbon dioxide. . . .Ethyl alcohol is formed in some quantity, nearly 5 per cent being produced at times from a 10 per cent dextrose solution. A limited amount of acid is also formed." One can thus calculate that about 1.9 mol of ethanol was produced per mol of glucose added. The formation of lactic acid was not reported.

The enzymic mechanism of hexose fermentation in this genus has been thoroughly examined in the strains, *Z. mobilis* ATCC 10988, as well as *Z. anaerobia* NCIB 8227 and *Z. anaerobia* NCIB 8777.

(i) Hexose catabolism in *Zymomonas* ATCC 10988. Lindner (104) established that his *Termobacterium mobile* was able to grow on and to ferment sucrose, glucose, and fructose with the formation of ethanol, CO_2, and lactic acid. In the 1920s, A. J. Kluyver (Delft) was very interested in fermentative bacteria. Sugar fermentations by *E. coli* and coliform bacteria, several saccharolytic clostridia, and propionic acid bacteria were being studied by several of his collaborators. It is therefore not surprising that he was interested in *Zymomonas,* an organism with a yeast-type fermentation. Kluyver and Hoppenbrouwers (89) confirmed that CO_2 was formed, but no H_2. The number of carbon sources for fermentation was limited to glucose, fructose, and sucrose. The fermentation of glucose was very active after 10 h at 30°C and was over after 2 days. Glucose was fermented to completion. In addition to ethanol, some lactic acid was produced. Traces of acetaldehyde were detected, but no other end products. Succinic acid was detected only when the fermentation occurred in the presence of yeast extract. It was not formed in a synthetic medium where the yeast extract was substituted by 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, and 0.05% $MgSO_4$. It is an artificial end product, arising from the reduction of amino acids in the yeast extract.

The molar fermentation equation, calculated from Kluyver and Hoppenbrouwer's data, is:

1 glucose → 1.8 ethanol + 1.9 CO_2 + 0.15 lactic acid.

Similar equations were later reported by other authors for strain NCIB 8938.

1 glucose → 1.93 ethanol + 1.8 CO_2 + 0.053 lactate
(Gibbs and DeMoss [67])

1 glucose → 1.58 ethanol + 1.7 CO_2 + 0.2 lactate
(Belaïch and Senez [17])

1 glucose → 1.63 ethanol + x CO_2 + 0.02 lactate
(Dawes et al. [44]).

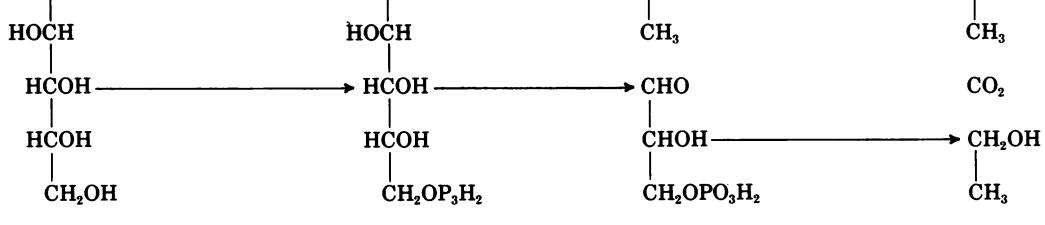
Slightly less ethanol and lactic acid accumulate when the bacteria are growing. It is thus not surprising that, in the early 1930s, the mechanism of the fermentation by *Zymomonas* was thought to be similar to that of *Saccharomyces*. That it was not, became obvious only many years later.

The above fermentation balance was confirmed by Schreder et al. (147). They found that 98% of the glucose is converted to CO_2 and ethanol. Small amounts of acetaldehyde, ace-

tylmethylcarbinol, acetic acid, lactic acid, and glycerol were found.

Neuberg and Kobel (119) examined some aspects of the intermediary carbohydrate metabolism in Lindner's organism. The following conversions were detected. Pyruvic acid is decarboxylated to acetaldehyde. Acetaldehyde can be trapped during the fermentation of glucose. Glucose-6-phosphate (G6P) and hexose diphosphate are dephosphorylated before fermentation. Phosphorylative processes occurred. The conclusion of Neuberg and Kobel that the fermentation of glucose occurs by way of the glycolysis was experimentally unfounded.

That the fermentation of glucose in *Zymomonas* does not follow the glycolytic pathway was discovered by Gibbs and DeMoss (66) with strain ATCC 10988. Almost all the activity of [1-^{14}C]glucose accumulated in CO_2; about half of the activity of [3,4-^{14}C]glucose was released in CO_2, and the remainder was found in ethanol. The breakdown by way of G6P-dehydrogenase and a C_2–C_3 split (phosphoketolase), as in *Leuconostoc*, was postulated but later found to be erroneous. These authors detected the presence of carboxylase and an acetoin-forming cell-free enzyme system. Gibbs and DeMoss (1954) discovered the basic mechanism of glucose and fructose breakdown in *Zymomonas*: it is a modification of the Entner-Doudoroff pathway. The fate of the majority of the individual C atoms is:



This assumption was tested and confirmed by Stern et al. (159) with a radiorespirometric method. $^{14}C_1$ and $^{14}C_4$ from glucose are rapidly released as CO_2. $^{14}C_2$ and $^{14}C_6$ are not converted into CO_2.

The presence of the Entner-Doudoroff mechanism was tested and confirmed later at the enzymic level by several authors. Cell-free extracts ferment G6P to ethanol and CO_2. The extracts contain phosphohexoisomerase, and an NAD-linked dehydrogenase for G6P. Fructose-1,6-diphosphate (F1,6P) is metabolized by way of aldolase and glyceraldehyde-3-phosphate NAD^+-linked dehydrogenase. All attempts to demonstrate the presence of phosphohexokinase were negative (134). Ribbons and Dawes (135) and Dawes et al. (44) detected a number of enzymes in *Zymomonas* NCIB 8938. Crude extracts with added ATP converted both glucose and gluconate into pyruvate. Hydrazine traps equivalent amounts of pyruvate and glyceraldehyde-3-phosphate from gluconate-6-phosphate. The conversion of glyceraldehyde-3-phosphate to pyruvate requires NAD^+, ADP, and inorganic phosphate. Indirect evidence strongly suggested the intermediate formation of 2-keto, 3-deoxygluconate-6-phosphate from gluconate-6-phosphate. It was, however, not isolated. The presence of hexokinase, gluconokinase, glucose dehydrogenase and pyruvate decarboxylase was established. G6P dehydrogenase is present and reduces either NAD^+ or $NADP^+$. Ethanol and triose phosphate dehydrogenases are NAD^+ specific. NADH oxidase activity is located mainly in the particulate fraction. NADPH oxidase was not detected. Neither gluconate dehydrogenase nor F1,6P aldolase was detected. However, Raps and DeMoss (134) had previously reported aldolase in the same strain. The presence of the latter enzyme is thus not clear. Sly and Doelle (153) presented a detailed study of the kinetics of G6P dehydrogenase with $NADP^+$. The values of optimal pH (8.7), optimal $MgCl_2$ concentration (10^{-2} M), and K_m (5×10^{-4} M for G6P and 3.6×10^{-5} M for $NADP^+$) were determined. The enzyme from *Zymomonas* was quite different from that of *E. coli* and some other microorganisms.

A number of the reactions of carbohydrate catabolism are summarized in Fig. 8. The oxidation-reduction balance of glucose and fructose breakdown is thus rather simple: it is probably maintained through NAD^+, rather than $NADP^+$, since NAD^+-$NADP^+$ transhydrogenase activity was not detected. For each molecule of hexose consumed, two NADH + H^+ are produced, one through G6P-dehydrogenase and one through glyceraldehyde-3-phosphate dehydrogenase. The reduced coenzyme is reoxidized largely by ethanol dehydrogenase and acetalde-

hyde, which is thereby reduced to ethanol, and to some minor extent by a weak lactate dehydrogenase. Figure 8 also shows that the overall ATP yield is one ATP per one glucose fermented. The complete enzymic pathway for the breakdown of gluconate appears to be present. Nevertheless, gluconate is neither a carbon nor an energy source (40). One reason may be that only one molecule of NADH + H^+ can be formed per molecule of gluconate added (from triose dehydrogenase), whereas two are required to reduce both molecules of pyruvate formed.

(ii) Hexose catabolism in *Zymomonas* NCIB 8777. Strain NCIB 8777 was compared with strain NCIB 8938 by McGill et al. (112) and McGill and Dawes (111). The former strain grew more slowly and with a smaller yield. The molar growth yield coefficients on glucose and fructose were 5.89 and 5.0, respectively. About 2% of the glucose was incorporated into the cells. The growth of this organism is thus not very efficient.

Both hexoses are fermented as follows:

$$1 \text{ glucose} \rightarrow 1.78 \text{ ethanol} + 1.88\ CO_2 + 3.10^{-5} \text{ acetaldehyde} + 0.011 \text{ (cell material } CH_2O)$$

The carbon balance of 90% indicates missing products.

$$1 \text{ fructose} \rightarrow 1.51 \text{ ethanol} + 1.55\ CO_2 + 0.02 \text{ acetaldehyde} + 0.054 \text{ glycerol} + x \text{ dihydroxyacetone}$$

The carbon balance is 77% without the three C compounds. Energetically wasteful products are formed during fructose metabolism, e.g., glycerol, dihydroxyacetone, and presumably others that remain to be identified.

The following enzymes were detected, in the order of decreasing specific activity: G6P dehydrogenase (NAD^+ or $NADP^+$ linked), ethanol dehydrogenase (NAD^+ linked), pyruvate decarboxylase, gluconate-6-phosphate (6GP) dehydratase plus 2-keto, 3-deoxygluconate-6-phosphate aldolase, triose phosphate dehydrogenase (NAD^+ linked), phosphohexose isomerase, glucokinase, fructokinase. The following enzymes are weak to very weak: phosphofructokinase, F1,6P-aldolase, NADH oxidase, 6PG dehydrogenase ($NADP^+$ specific), NADPH oxidase, gluconokinase, glucose dehydrogenase. The following enzymes are absent: $NADP^+$-NAD^+ transhydrogenase, transketolase, phosphoketolase, and transaldolase. The key intermediate, 2-keto, 3-deoxygluconate-6-phosphate, was detected by paper chromatography, but it was neither isolated nor identified more closely.

For growth and enzyme biosynthesis, DNA and RNA are required. The deoxyribose moiety is derived from ribose-5-phosphate (R5P). In most organisms, the latter compound is produced by the pentose phosphate pathway. However, in *Zymomonas* NCIB 8777, this pathway cannot be active because transketolase and transaldolase activities were not detected. The function of the weakly active 6PG dehydrogenase may be to provide R5P.

Zymomonas strains NCIB 8938 and NCIB 8777 are the only bacteria currently known to utilize the Entner-Doudoroff pathway anaerobically in association with a pyruvate decarboxylase. This is almost certainly the case for all other strains in this genus, although they have not yet been examined in this respect. The

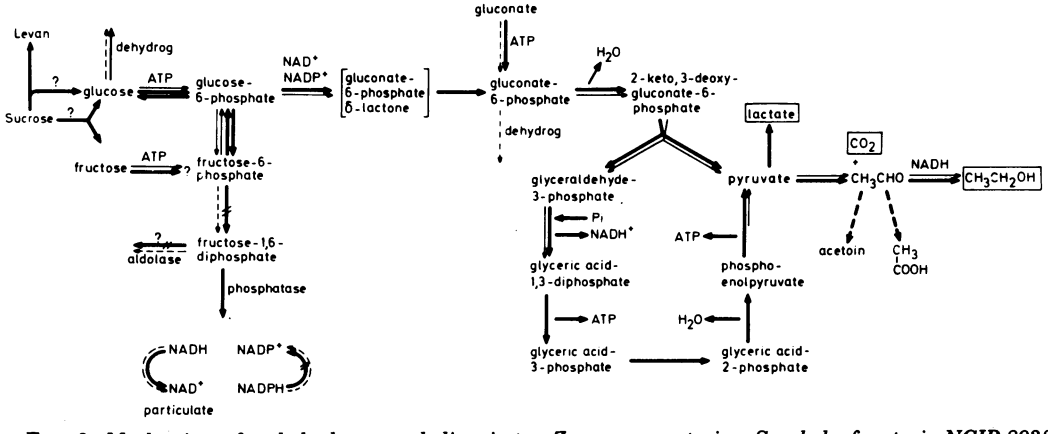


FIG. 8. *Mechanism of carbohydrate catabolism in two Zymomonas strains. Symbols: for strain NCIB 8938* (——), *active;* (- - - -), *weakly active; for strain NCIB 8777* (——), *active;* (----), *weakly active.*

differences in the enzymic equipment for carbohydrate catabolism between both strains are small. They have to be considered only as differences between individual strains and not as criteria or proof for species differentiation.

Metabolism of sucrose: fermentation and levan formation. Strains able to grow on and ferment sucrose have been isolated mainly from fermenting plant saps in tropical and subtropical areas and from infected British beer. Strains unable to metabolize sucrose have been isolated in the United Kingdom mainly from infected beers, sick cider and perry, and apple pulp (see Table 5). The metabolism of sucrose is nearly always tested in a growth medium. Fermentation to ethanol and CO_2 is accompanied by growth. Sixteen out of 33 *Zymomonas* strains, isolated by one of us from Zaïrese palm wines, neither grow on nor ferment sucrose.

From the numerical analysis of 138 phenotypical features in 38 *Zymomonas* strains, we found that the ability to metabolize sucrose is not correlated with any other feature (50). In addition, the fermentation of sucrose seems to be an inducible strain-specific phenomenon (see above and reference 17, 36, 89, 137) without taxonomic value.

The first step of sucrose breakdown should almost unavoidably yield glucose or fructose or both. Important aspects of the enzymic mechanism were clarified by Dawes et al. (45, 136). They discovered that *Zymomonas* strain NCIB 8938 produced levan from sucrose, but not from glucose or fructose or a mixture of both; 640 g of sucrose in 32 liters of medium gave 4.5 g of dry polymer. The yield range was less than 2%. The *Zymomonas* levan would appear to be a typical member of the bacterial levan family. It has a molecular weight of about 10^7. It contains only fructose, consisting thus of about 60,000 fructose units. The major linkage is 2,6; many (probably all) units are in the furanose ring structure; 2,1 linkages are present at branchpoints; the configuration is probably β. During the synthesis of levan, glucose is utilized faster than the fructose moiety. Levan formation stops when the sucrose concentration is 0.5 mg/ml. Only free fructose is left, and this continues to be metabolized by the cells without levan formation. Levan is synthesized by crude extracts of *Zymomonas* from sucrose and from raffinose without added primer; the majority of the sucrose is hydrolyzed to glucose and fructose. The levan sucrase differs from those previously studied in *Acetobacter levanicum* and in *Bacillus subtilis*.

Two strains B70 and NCIB 8227 from infected British beer also produce levan when grown in a sucrose-containing medium (36). Unfortunately, the chemical proof of the identity of this material was incomplete, and no quantitative data were given on the amount of levan produced and on the importance of the levan sucrase pathway in the breakdown of sucrose by both strains. Thus, so far, three *Zymomonas* strains appear to produce levan from sucrose. It may very well be that all sucrose-fermenting *Zymomonas* strains behave in the same way.

The results of Dawes et al. (45) show that less than 10% of the sucrose is metabolized by a levan sucrase with the formation of levan and glucose. The major pathway of sucrose catabolism in *Zymomonas* starts after a hydrolytic split to glucose and fructose. Dawes et al. (45) have not established whether an independent invertase is present. They suggested tentatively that a levan sucrase can account for both levan formation and sucrose hydrolysis by the reaction:

$$n \text{ sucrose} + \text{acceptor} \rightarrow \begin{matrix}\text{levan} + n \text{ glucose} \\ \text{or} \\ n \text{ fructose}\end{matrix}$$

If the acceptor is fructose, levan is formed; if the acceptor is water, sucrose is hydrolyzed. Phosphorolysis by way of the reaction

$$\text{sucrose} + P_i \rightleftharpoons \text{glucose-1-phosphate} + \text{fructose}$$

plays no part in the sucrose degradation by *Zymomonas*.

The average molar growth coefficient of *Zymomonas* strain NCIB 8938 on sucrose, $Y_s = 7.4$, is appreciably lower than those for the equivalent concentrations of glucose, fructose, or glucose plus fructose (average, about 9.2). Molar growth yields on sucrose are erratic and low, due to the formation of levan (45).

Two important problems in sucrose metabolism remain to be solved: (i) Is the hydrolysis of sucrose effected by a levan sucrase or by a separate invertase? (ii) Is the acquisition of sucrose catabolism due to the induction of new enzymes, eventually accompanied by levan production, or to the selection from the population of some cells with constitutive enzymes?

Other carbon sources. None of the following C sources supported growth of the *Zymomonas* strains of our collection: starch, dextrin, raffinose, D-trehalose, maltose, lactose, D-cellobiose, D-galactose, D-mannose, L-rhamnose, L-sorbose, D-arabinose, L-arabinose, D-ribose, D-xylose, D-sorbitol, D-mannitol, dulcitol, adonitol, erythritol, glycerol, ethanol, salicin, and the sodium salts of D-galacturonic, succinic, DL-malic, tartaric, DL-lactic, pyruvic, and citric acids.

According to Millis (115), *Zymomonas* strain

L192 (= ATCC 10988) grew on raffinose but formed no gas; five other strains examined by her did not grow. Dadds et al. (36) stated that growth on raffinose could be induced in two *Z. anaerobia* strains and in *Z. mobilis* B70.

Sorbitol is said by Millis (115) to be a carbon source permitting growth without gas formation in five of the six strains examined. Growth on sorbitol occurred neither in another strain of *Z. anaerobia* (29) nor in the strains B70 and NCIB 8938 (36). As in the cases of raffinose and sucrose utilization, inducibility is a plausible hypothesis.

Amino acids as sole C source are not fermented; they do not support growth when glucose is absent (15).

Formation of acetaldehyde. Acetaldehyde can be trapped during the fermentation of glucose. Millis (115) attributed the full fruity aroma that develops in cider sickness to the liberation of acetaldehyde. This compound, together with variable amounts of hydrogen sulfide, contributes to the unpleasant taste and smell of sick cider. Barker (11) suggested that "the designation (cider-) sickness must be considered as a general one, applying to a type of fermentation distinguished by the breakdown of certain sugars to yield not only ethanol but also aldehydes—especially acetaldehyde—in sufficient quantity to justify its appellation as 'aldehydic' fermentation." In cask or keg beers, *Zymomonas* infection produces an unpleasant odor of rotten apples due to traces of H_2S and acetaldehyde (9, 151). *Zymomonas* produces high acetaldehyde concentrations in beer when brewing yeast or *Gluconobacter* is absent (34). There is a linear correlation between the number of *Zymomonas* cells present and the amount of acetaldehyde produced: 10^{10} cells per liter of beer produce about 80 mg of acetaldehyde. The results of a laboratory experiment on the production of acetaldehyde by *Zymomonas* strain NCIB 8227 in a pale-ale in the presence of *Saccharomyces cerevisiae* and *Gluconobacter oxydans* NCIB 8131 are summarized in Table 11.

TABLE 11. *Production of acetaldehyde by Zymomonas NCIB 8227 in the presence of Saccharomyces cerevisiae or Gluconobacter oxydans NCIB 8131 or both*[a]

Initial concn (cells/ml)	Aldehyde production (mg/liter) with initial *Zymomonas* concn (cells/ml) of:	
	0	2×10^6
Yeast—0		
Gluconobacter added:		
0	3.0	78.3
2×10^6	5.8	61.4
Yeast—2×10^6		
Gluconobacter added:		
0	10.1	17.7
2×10^6	6.4	24.5

[a] Data from reference 34.

Acetaldehyde flavors have only been noted in beers containing glucose and fructose. High acetaldehyde levels and *Zymomonas* infection have never been observed in lager beers (34). Schreder et al. (147) made the interesting observation that limited aeration of the fermenting mass increased the acetaldehyde formation.

Formation of acetylmethylcarbinol. Kluyver and Hoppenbrouwers (89) detected traces of acetylmethylcarbinol in *Zymomonas* cultures. Tankó (165) artificially increased the production of acetoin by this strain by carrying out fermentations of glucose or sucrose in the presence of added acetaldehyde. Twenty to 35% of this substrate was converted into acetoin. No 2,3-butyleneglycol was detected. Schreder et al. (147) showed an increased formation of acetaldehyde and acetylmethylcarbinol by increased aeration. We found that the 42 strains of our collection all produce traces of acetylmethylcarbinol. Shimwell (150) and Millis (115) did not detect this compound. These negative results are probably due to different test conditions or sensitivity or both.

The enzymic mechanism of acetoin formation in *Zymomonas* is not known. In *Enterobacter* it is:

$$\text{pyruvate} + \{\text{aldehyde}\}\text{:TPP} + H^+ \rightarrow \alpha\text{-acetolactate} + TPP^+$$

and

$$\alpha\text{-acetolactate} \rightarrow \text{acetoin} + CO_2$$

In yeast, plants and animals it is:

$$\text{acetaldehyde} + \{\text{aldehyde}\}\text{:TPP} + H^+ \rightarrow \text{acetoin} + TPP^+$$

It is interesting to recall that many acetic acid bacteria produce acetoin both by the α-acetolactate and by the acetaldehyde pathways (46).

Molar growth yield. Bauchop and Elsden (14) determined the quantitative relation between the growth yield of some anaerobic bacteria and the amount of energy supply. As long as the latter is the growth-limiting factor, the dry weight of cells produced is proportional to the amount of energy source added, or better, to the ATP yield of the catabolic reactions. The growth yield coefficient $Y_{substrate}$ is the gram (dry weight) produced per mole of substrate added. The ratio Y_{ATP} is the dry weight of cells,

expressed in grams, produced per mole of ATP formed. For *Streptococcus faecalis* and *S. cerevisiae*, the average Y_{ATP} is 10.8 (14). Until rather recently it has been assumed that all anaerobic microorganisms have about the same efficiency at converting the catabolic energy into cellular material. This appears not to be the case, as several higher Y_{ATP} values are now known, e.g., Y_{ATP} = 18 to 20 for *Lactobacillus casei* (54).

With *Zymomonas*, $Y_{glucose} = Y_{ATP}$ because of the mechanism of glucose catabolism. The molar growth yields of *Zymomonas* with glucose or fructose as energy source have been determined by several authors and found to vary considerably, from 3.5 to 9.3 (Table 12). Growth yields and growth rates of anaerobic cultures of strain NCIB 8938 were determined in three media (17) (see Table 15). Aeration of the cultures did not modify either the yield or the division time. The growth yield of *Zymomonas* is thus much less efficient, as with most other anaerobes, even in rich media where the energy substrate is certainly the limiting factor. There is no significant difference in Y_{ATP} between strains labeled *mobilis* and *anaerobia*. The low values of Y_{ATP} in batch cultures may be a function of the specific growth rate μ, the maintenance energy m_e, the cell composition, etc. (54, 160, 161). The differences in Y_{ATP} values observed for one organism by several authors (Table 12) may be due to differences in specific growth rate caused by different growth conditions and medium composition (161).

The growth of *Zymomonas* NCIB 8938 was studied calorimetrically by Senez and collaborators (15, 18, 149). The rate of heat evolution dQ/dt was measured with an isothermic calorimeter. This strain, inoculated in 0.4% yeast extract, 0.4% peptone, and 0.05 to 0.2% glucose in 0.025 M tris(hydroxymethyl)aminomethane-maleate buffer, pH 6.8, grows quickly and exponentially until only 0.4 mg of glucose per ml (0.04%) remains; then growth and heat production slow down and stop when all glucose is completely exhausted. The complete reactions of glucose decomposition are:

$$\text{Glucose} + \text{ADP} + P_i \rightarrow 1.8\ \text{ethanol} + 1.8\ CO_2 + 0.2\ \text{lactate} + \text{ATP} + H_2O$$

and

$$\text{ATP} + H_2O \rightarrow \text{ADP} + P_i$$

The molar growth yield $Y_{glucose}$ is a constant during the entire growth period. The change in enthalpy of the sum of the above reactions is −32.62 kcal/mol of glucose consumed. The heat released from glucose during growth agrees perfectly with the theoretical enthalpy calculated for the equation of Kluyver and Hoppenbrouwers (89) from the standard enthalpies of formation for aqueous glucose (−302.03), ethanol (−68.85), CO_2 (−98.69), and lactic acid (−164.02; −0.85 heat of neutralization). The amount of cells produced and of heat evolved depend linearly on the quantity of glucose metabolized:

$$1\ \text{mol glucose consumed} \rightarrow 6.5\ \text{g dry cells} + 32.7\ \text{kcal}$$

If the dry, living cell material is roughly represented by (CH_2O) the total equation of growth becomes:

$$\text{Glucose} + (\text{carbon source}) \rightarrow 1.8\ \text{ethanol} + 1.8\ CO_2 + 0.2\ \text{lactic acid} + 0.22\ (CH_2O) + 32.7\ \text{kcal}$$

When cultivated in the usual complex medium containing large quantities of peptone and yeast extract, *Zymomonas* utilizes glucose as the exclusive energy source for growth and has no detectable endogenous metabolism. The bacteria do not accumulate appreciable amounts of intermediates such as glycogen or phosphorylated polymers from glucose.

One of the important conclusions on the carbohydrate metabolism of *Zymomonas* is that, when growing in a complex medium, 98% of the glucose consumed is converted in ethanol, CO_2, ATP, and heat, and only 2% is used as building material for the cell. This situation is rather similar to what happens in the acetic acid bacteria, where 85% of the glucose is oxidized to gluconate, 15% by way of the pentose phosphate pathway (49). Nevertheless, the 2% glucose incorporated in *Zymomonas* produces 48% of the cellular carbon (17); the rest is derived from yeast extract components or peptone or both. Amino acids and/or the various organic constituents of peptone and/or yeast extract are not utilized as an energy source for growth, but only as building blocks for biosynthesis.

TABLE 12. *Molar growth yields of Zymomonas*[a]

Strain	Growth yield coefficients, Y	References
ATCC 10988	$Y_{glucose}$ = 4.32 − 9.32	14, 16, 17, 18, 21, 45, 62, 97
	$Y_{sucrose}$ = 5.35 − 8.0	45
NCIB 8777	$Y_{glucose}$ = 5.89	111
	$Y_{fructose}$ = 5.0	111
NCIB 8227	$Y_{glucose}$ = 3.4 − 3.88	21, 42
Z1-Z8	$Y_{glucose}$ = 3.48 − 4.12	21

[a] For definition of Y, see text.

Uncoupled growth. In the previous section, we have seen that not all ATP produced from sugar catabolism by *Zymomonas* is used for growth processes. This phenomenon is but one aspect of an uncoupled growth. One of its definitions is that the rate of substrate dissimilation per unit weight of organism is independent of the growth rate.

Several aspects of uncoupled growth in *Zymamonas* NCIB 8938 were studied by Belaïch et al. The discussion below is based mainly on their results (16, 19, 97). One of the factors necessary for uncoupling growth is lack of pantothenate. This compound apparently can not be synthesized by *Zymomonas* strains. As a component of coenzyme A (CoA), it is required for the activity of various transferases and is thus of very great importance in biosynthesis. With limiting pantothenate concentrations (5×10^{-7} mg/ml) in defined media, the molar growth yield on glucose falls to $Y_{glucose} = 2.5$. The amount of glucose metabolized is constant at 0.991 mmol of glucose per min per g (dry weight) and independent of the rate of growth, the nature of the medium, and the concentration of the growth factor pantothenate.

The explanation of uncoupled growth pivots upon the fate of ATP produced by fermentation. Lazdunski and Belaïch (97) measured changes in the ATP pool. The ATP content of the cell goes through a weak maximum at the onset of growth and remains constant during the exponential phase; depending on the growth medium used there is between 1 and 4 μg of ATP/mg (dry weight). During this growth phase, energy production and consumption seem to be well balanced. When the last generation of cells is formed, the ATP content drops to about 0.4 μg/mg (dry weight). The constancy of the ATP pool during exponential growth has been observed in other bacteria, e.g., in *E. coli* (83).

In conditions of poor biosynthesis and growth, e.g., in synthetic and minimal media poor in pantothenate, the rate of glucose breakdown remains the same, and the ATP pool in the cell is twice as large. When growth is stopped suddenly by chloramphenicol, glucose breakdown continues, the rate of heat production remains the same, and the ATP pool almost doubles. The level of ATP in the cell depends on (i) its rate of formation and (ii) its rate of disappearance. According to Belaïch's data, the rate of fermentation, and thus of ATP formation, is rather constant. ATP could disappear in several ways, by biosynthesis, by ATPase action, by leakage, etc. There may be still other reasons for apparent growth uncoupling, such as extra energy required to maintain an organism at low growth rates (126) or leakage of important metabolic intermediates from the cells. Stouthamer and Bettenhaussen (161) stressed the underestimation of maintenance energy and its influence on molar growth yields. They think that coupling between energy production and growth rate is much tighter than has been supposed hitherto. A coupled growth is of maximal efficiency when ATP is produced only when needed, and in the amount required, for biosynthetic mechanisms. Regulation is thus required, such as happens, e.g., in glycolysis in *E. coli*, where the energy charge of the adenylate system is increased whenever ATP is used by biosynthetic mechanisms. Phosphofructokinase is activated by ADP or AMP and inhibited by phosphoenolpyruvate; pyruvate kinase is activated by AMP and F1,6P. We postulate here that the uncoupled growth of *Zymomonas* occurs because these or similar control mechanisms either do not exist or function very poorly. The data of Forrest (62) may suggest some energy coupling in *Zymomonas* between 24 and 33°C, although the eventual nature and mechanism is unknown. Within this temperature range, the rate of glucose consumption and the rate of specific cell growth both increased with the same energy of activation, 11.1 kcal/mol. Above this temperature (from 33 to 39°C), the rate of glucose consumption continues to increase, but the specific growth rate dropped. The coupling between anabolism and catabolism is thus not very effective above 33°C.

The scenario for the energy flow in *Zymomonas* may thus be summarized as follows. Glucose catabolism occurs at a constant rate of one ATP to one glucose, and the waste material comprises ethanol, CO_2, and lactate. The composition of the medium and the state of biosynthesis for the construction of the daughter cell seem to be of little importance for the main ATP production. If the medium and conditions are suitable for growth and division of the cell, the biosynthetic pathways will use the ATP produced; if the rate of growth decreases or stops, the energy source will continue to be utilized until it is exhausted. Lazdunski and Belaïch (97) suggested from some preliminary experiments that the excess of energy in ATP is removed by an adenosine triphosphatase.

The apparent uncoupled growth of *Zymomonas* NCIB 8938 on sucrose is partially accounted for by the production of levan.

Aerobic growth and metabolism. *Zymomonas* is not a strictly anaerobic organism. *Zymomonas* strains NCIB 8938 can grow in the presence of oxygen (44). Belaïch and Senez (17) prepared aerobic cultures of the same strain on a reciprocal shaker. The growth yield and divi-

sion time were not different from an anaerobically grown culture.

Lindner (104) and Kluyver and Hoppenbrouwers (89) observed that glucose fermentation in semianaerobic conditions gave about one-third the ethanol yield of strict anaerobiosis, a finding attributed to a very powerful respiratory system. This phenomenon was examined quantitatively by Schreder et al. (144–147). Their results are summarized in Table 13. In the presence of excess oxygen, *Zymomonas* did not break down glucose (in contrast to results of other authors). When limited amounts of O_2 were supplied, somewhat less ethanol was formed, and there was a small increase in acetaldehyde. Changes in other products were very small and nearly unimportant for the economy of the cell. The respiratory activity of *Zymomonas* is present in anaerobically grown cells. The oxidative capacities of the cells seem to be affected considerably by the medium on which they have been grown (17). Both aerobically and anaerobically grown cells oxidize glucose (44). The latter cells have a $Q(O_2)$ of 60 to 80 and take up 0.5 to 0.8 mol of O_2 per mol of glucose added and the former ones have a $Q(O_2)$ of 100 to 140 and use 0.7 to 1.5 mol of O_2 per mol of glucose. The aerobic dissimilation of glucose corresponds to the equation: 1 glucose + 1 O_2 → 1 ethanol + 1.7 CO_2 + 1 acetate + 0.2 lactate (17). The mechanism seems to be limited to the oxidation of ethanol to acetic acid. Even anaerobically grown *Z. anaerobia* is able to oxidize ethanol without a lag (41). The transfer of electrons by the respiratory chain is apparently not coupled with oxidative phosphorylation. According to Gonçalves de Lima et al. (70), strains of *Zymomonas mobilis* var. *recifensis* appear to be more aerobic than other *Zymomonas* strains. However, several strains of the subspecies *mobilis* in our collection were as aerotolerant. According to Magalhães Neto et al. (108), strain Ag 11 oxidizes the following sugars [$Q(O_2)$ values are given in parentheses]:fructose (38), glucose (35), sucrose (27), xylose (14), galactose (5), mannose (3), maltose (3), and endogenous (1). Lactose and a few organic acids were not oxidized.

The species name of *Z. anaerobia* suggests that the organism is either strictly anaerobic or at least more anaerobic than *Z. mobilis*. McGill and Dawes (112) observed that this organism is facultatively aerobic, contrary to earlier reports (150, 151), which classified the organism as "microaeroduric." *Z. anaerobia* NCIB 8227 grows when aerated vigorously on a gyratory shaker. The yield of these cells and their rate of growth are only 50% of anaerobically grown cells (38). It is likewise our own experience that, as far as anaerobic response is concerned, named *mobilis* and *anaerobia* strains grow in the same fashion. To stress the anaerobic features with a species name is not justified.

The presence and possible role of the tricarboxylic acid cycle in *Zymomonas* NCIB 8227 has been examined by Dawes et al. (43). Four enzymes of the tricarboxylic acid cycle have been detected: citrate synthase, aconitase, isocitrate dehydrogenase, and malate dehydrogenase. Aeration of intact cells does not induce higher activities of these enzymes. The specific activities of the *Zymomonas* enzymes are similar to those reported for anaerobically grown *E. coli*, with the exception of malate dehydrogenase. The following enzymes were not detected: succinate-, α-ketoglutarate-, and glutamate dehydrogenases; succinyl-CoA-, δ-amino-laevulinate-, and malate synthetases; fumarate hydratase, succinate thiokinase, and isocitrate lyase. Small amounts of all the intermediates of the cycle were detected. Whether they are formed by the cycle enzymes, present in too low activities to be detected, or by some other mechanism is not known.

The fragments of the tricarboxylic acid cycle in *Zymomonas* may be correlated with the presence of the Entner-Doudoroff pathway. Both mechanisms are typical of aerobic organisms, and their presence in preponderantly anaerobic organisms is surprising. They suggest to us

TABLE 13. *Effect of oxygen on the fermentation of Zymomonas*[a]

Compounds formed	mol of product per mol of glucose fermented with added O_2 (mol):				
	0	0.4	0.6	1.5	∞
CO_2	1.94	1.95	1.96	1.91	0
Ethanol	1.99	1.92	1.91	1.70	0
Acetaldehyde	Trace	0.024	0.021	0.17	0
Acetoin	Trace	0.01	0.01	0.04	0
Acetic acid	0.01	0.06	0.06	0.03	0
Lactic acid	0.002	0.002	0.002	0.004	0
Glycerol	0.004–0.03	0.008	0.009	0.019	0

[a] Data from Schreder et al. (147).

that *Zymomonas* is evolutionarily derived from aerobic ancestors, by decrease or loss of several tricarboxylic acid cycle enzymes and electron transport enzymes. It is significant that strictly aerobic, polarly flagellated *Gluconobacter* strains likewise possess the Entner-Doudoroff mechanism and fragments of a tricarboxylic acid cycle. We suggest this evidence may constitute two more indications that *Zymomonas* and the acetic acid bacteria are remote relatives.

Cytochromes. *Z. mobilis* NCIB 8938 displays cytochrome bands at the temperature of liquid nitrogen (17). Aerobically grown cells have the type *c* cytochrome 550 (β band 520 and 528) and the type a_2 cytochrome 620. Anaerobically grown cells have in addition the type *b* cytochrome 560. Low concentrations of cyanide (10^{-4} M) inhibit the respiration of this strain completely. *Z. mobilis* probably respires through a complete respiratory chain, in which cytochrome *c* and a cytochrome oxidase of the a_2 type are constitutive (17).

Aerobically and anaerobically grown cells of *Z. anaerobia* both contain a type *b* cytochrome and traces of *c* cytochrome (112). These differences between both strains of *Zymomonas* are to be considered as minor and individual, rather than as criteria for species differentiation.

We are not aware of a systematic comparative investigation of cytochromes in *Zymomonas*. A comparative study in many strains of *Acetobacter* and *Gluconobacter* showed that a cytochrome pattern has no taxonomic significance (118).

The pigment P-503. A pigment absorbing at 503 nm has been detected in different spectra of yeast and several bacteria (74, 99). This pigment is not a hemoprotein; it displays the same properties as tetrahydroporphyrin (95, 124). Kropinski et al. (93) detected this pigment in *Zymomonas* ATCC 10988; judging from their data, there is not very much present. The eventual role of this pigment in the metabolism of *Zymomonas* is not clear. In *E. coli* this pigment may have some function in electron transport, because it is in kinetic equilibrium with flavoprotein (124).

Amino Acid Metabolism

Amino acid requirements. The first nutritional studies were carried out with strain NCIB 8938 by Belaïch and Senez (17). A mixture of 20 amino acids, or even NH_4Cl, could partially replace yeast extract and supported growth in the presence of glucose and Ca-pantothenate in the synthetic and minimal media, respectively. The molar growth yields in the latter media are only about half the value from the complex medium; the generation time and the cellular carbon from glucose both increase. Ethanol formation remains approximately the same (Table 14). The growth of *Zymomonas* NCIB 8227 is stimulated by individual amino acids (Table 15), by NH_4Cl, and by groups of amino acids, but the complete set of amino acids is necessary to obtain a growth yield comparable to growth on peptone (42) (Table 16). From all the amino acids tested individually as a sole source of nitrogen for growth of *Zymomonas* NCIB 8227, either arginine, tryptophan, glutamic acid, or cystine produced the best growth (23, 42) (Table 15). Maximum growth was supported by either glutamic acid, lysine, hydroxyproline, or cysteine with a *Zymomonas* isolate tested by Richards and Corbey (137).

To establish the effect of amino acids on the growth of 38 *Zymomonas* strains, Van Pee et al. (174) used the liquid synthetic medium of Belaïch and Senez (17), but added a mixture of 10 vitamins (23), guanine, adenine, hypoxanthine, cytosine, and uracil. This medium covered the nutritional requirements of all *Zymomonas* strains. Five transfers were made, and the growth was measured. The omission of each of the 20 amino acids separately from the amino acid mixture did not depress growth of *Zymomonas* completely; i.e., none of the 20 acids is essential to *Zymomonas* when other amino acids are present. The growth of 10 strains out of 38 showed no marked reduction upon withdrawal of any of the amino acids from the synthetic medium. In individual *Z. mobilis* subsp. *mobilis* strains, up to four amino acids reduced growth, whereas in the most exacting strain, *Z. mobilis* subsp. *pomaceae* ATCC 29192, the withdrawal of any one of six amino acids caused a reduction of growth (Fig. 9). From Fig. 10 it is seen that valine and leucine reduced growth in 18 and 8 strains, respectively. The claim of Bexon and Dawes (23), that *Z. anaerobia* NCIB 8227 is more exacting for amino acids than *Z. mobilis*, is contradicted by our results.

The above information concerns the effect of amino acids on growth and growth yield. Data from Belaïch and Senez (17) show that about half of the cellular carbon is derived from glucose. Clearly, the other half can come from one or more amino acids. Some of them, thus, not only serve as a nitrogen source, but also as a carbon source. Therefore, we determined the amino acid uptake by *Zymomonas* ATCC 10988 growing in the synthetic medium of Bexon and Dawes (23) (J. De Ley, J. Swings and J. Van Beeumen, unpublished data). Three amino acids only, aspartic acid, serine,

TABLE 14. *Growth characteristics of Zymomonas NCIB 8938 in complex, synthetic and minimal media*[a]

Medium	Nitrogen source	Molar growth yield (Y_G) (g [dry cells] per mol of glucose fermented)	Generation time (h)	Ethanol formed (mol/mol of glucose)	Cellular carbon derived from glucose (%)
Complex	Peptone + yeast extract	7.95	1.90	1.57	48
Synthetic	20 Amino acids	4.98	2.62	1.52	62
Minimal	NH_4Cl	4.09	3.0	1.50	100

[a] Data from reference 17.

TABLE 15. *Efficiency of individual amino acids for growth of Zymomonas NCIB 8227*[a]

Amino acid (1.35%)[b]	Net growth (μg [dry wt]/ml)
Gly	23
Ile, Leu, Cys	25
Ser	26
Thr	28
Hyp	32
His	37
Val	43
Tyr	51
Met	53
Phe	63
Ala	73
Cys—Cys	76
Glu	83
Trp, Arg	87

[a] Data from reference 40.

[b] Added to a medium containing 2% glucose, inorganic salts, biotin, and lipoic acid (each, 20 μg/ml).

TABLE 16. *Different nitrogen sources for growth of Zymomonas NCIB 8227*[a]

Supplement (each amino acid at 0.067%)[b]	Net growth (μg [dry wt]/ml)
Ammonium chloride	57
Groups of amino acids	
Arg + Cys + Cys—Cys + Met	51
Ile + Leu + Val	48
Phe + Trp + Tyr	60
Asp + Glu + His + Thr	53
Ala + Gly + Hyp + Ser	58
All amino acids	121
All amino acids + NH_4Cl	139
Synthetic medium (17)	146
Peptone	172

[a] Data from reference 42.

[b] Added to a basal medium containing 2% glucose, inorganic salts, biotin, and lipoic acid (20 μg/ml each).

and cysteine, were taken up in appreciable amounts. Six other amino acids were not taken up at all from the medium (Tyr, Pro, Phe, Glu, Ala, Trp). From the remaining amino acids, small amounts were consumed, but less than

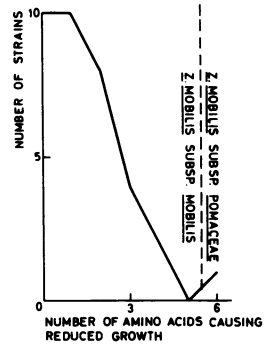


FIG. 9. *Number of amino acids that reduced growth in individual Zymomonas strains.*

15% of the amount supplied (Table 17).

There is evidence that, in many bacterial genera and species, the amino acid requirements are strain specific (31, 45, 113, 140). In *Zymomonas* also many amino acids stimulate growth, and the degree of stimulation varies from strain to strain. Amino acid requirements are therefore of little taxonomic use in *Zymomonas*.

Formation of higher alcohols. Plant juices, beers, and ciders, infected with *Zymomonas*, develop typical fruity aromas, which are very important for the commercial value of these products. The aromas of common alcoholic beverages are largely due to esters and fusel alcohols. Therefore, Verachtert and collaborators (21, 22, 133, 175) determined the fusel alcohols produced by *Zymomonas*. These bacteria produce about 0.6 mg of total fusel alcohols per g of glucose fermented; this is about 40 times less than *Saccharomyces* in the same conditions. The most important components of the fusel alcohol fraction are propanol and isoamyl alcohol in *Zymomonas* (Table 18), and isobutanol and isoamyl alcohol in *S. cerevisiae*. No considerable differences in fusel alcohol production were observed among nine *Zymomonas* strains studied. The nature and the very small amount of fusel alcohols formed by resting *Zymomonas* cells in glucose solution suggest that they are derived mainly from amino acids and much less

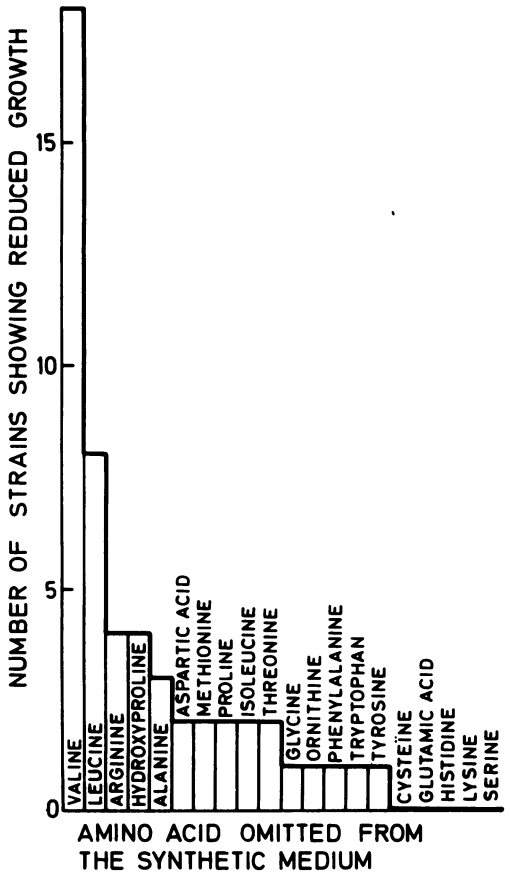


FIG. 10. *Number of Zymomonas strains showing reduced growth by omission of individual amino acids from the synthetic medium. Thirty-eight strains were tested.*

from carbohydrate metabolism. Direct proof was obtained by the addition of a variety of compounds. The addition of α-ketobutyric acid, threonine, propionaldehyde, and homoserine, in that order, stimulates the formation of propanol. Isobutyraldehyde and valine activate isobutanol production. Isoleucine increases the formation of D-amyl alcohol. Forssman (63) demonstrated that DL-methylethylacetaldehyde is reduced to amyl alcohol by actively fermenting *Zymomonas*. α-Ketoisocaproic acid, isovaleraldehyde, and leucine stimulate the formation of isoamyl alcohol. Oxaloacetate appears to stimulate the production of several fusel alcohols, on the one hand by decarboxylation, on the other hand by transamination to aspartic acid. The general pathways existing in yeasts and in *Zymomonas* for the formation of fusel alcohols are represented in Fig. 11.

Amino acid decarboxylases. Of 38 *Zymomonas* strains tested, 31 contain L-arginine decarboxylase, 18 contain L-lysine decarboxylase, and 4 contain L-ornithine decarboxylase (50). Five do not possess any of the above-mentioned three amino acid decarboxylases. The tests were read after 10 to 15 days of incubation.

Growth Factor Requirements

Cider sickness strains B and C required pantothenic acid, riboflavin, biotin, and nicotinic acid (11).

Zymomonas NCIB 8938 required 50 ng of pantothenate per ml only for growth in synthetic or minimal medium (17). Van Pee et al. (174), however, found with two other subcultures, 410 and ATCC 10988, of the same organism, that both pantothenate and biotin are required. In synthetic or minimal medium containing excess pantothenate, the molar growth yield and the growth rates of *Zymomonas* NCIB 8938 were only about half of the values in the complex medium. Both remained constant over a series of successive transfers. Belaïch and Senez (17) tried unsuccessfully to restore the molar growth yield to the level of the complex medium by separate or simultaneous addition of several compounds, such as ascorbic acid, menadione, nicotinic acid, etc.

Stephenson et al. (158) showed that the nutritional requirements of *Zymomonas* NCIB 8227 did not remain constant. When first checked in 1970 (23), this strain required lipoic acid or biotin for growth. Upon reexamination of three subcultures of the same strain a few years later, the vitamin requirement had changed to pantothenate (Table 19) (158). The same authors reported that another *Zymomonas* strain showed a requirement for *p*-aminobenzoic acid, folic acid, biotin, and cyanocobalamine upon isolation. Five months later, one of the subcultures showed a requirement for Ca pantothen-

TABLE 17. *Uptake of amino acids by Zymomonas ATCC 10988 during growth in the medium of Bexon and Dawes (23)*[a]

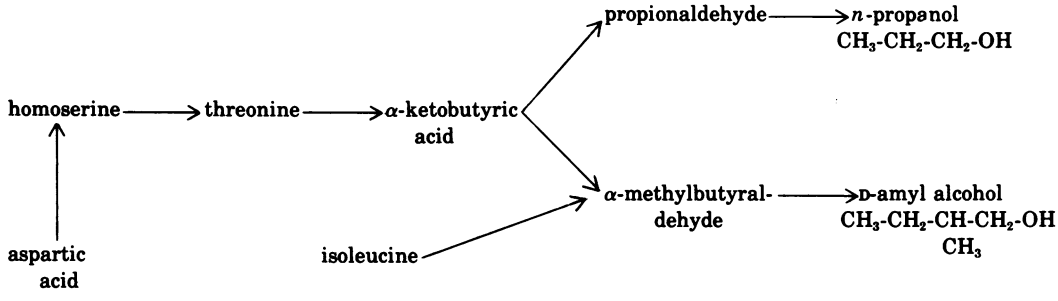
Amino acids taken up from the medium for:		Amino acids not taken up from the medium
50% of initial concn	15% or less of initial concn	
Asp	Arg	Tyr
Ser	Gly	Phe
Cys	Ile	Ala
	Leu	Pro
	Orn and/or Lys	Glu
	His	Trp
	Met	
	Val	
	Thr	

[a] Unpublished data from J. De Ley, J. Swings, and L. Van Beeumen.

TABLE 18. *Formation of fusel alcohols by Zymomonas and Saccharomyces*[a]

Organisms	State of cells	Alcohol formed (μg/ml)					
		Propanol	*i*-Butanol	*n*-Butanol	D-Amyl alcohol	Isoamyl alcohol	Avg total, higher alcohols
Zymomonas	Growing culture	4.1-21.4	Traces	Traces	Traces	6.6-17.6	25.96
	Resting cells in 5% glucose solution, pH 6.4	Traces	Traces	0	0	0.81	0.81
Saccharomyces cerevisiae	Growing culture	13.71	779.2	0	60.22	95.65	948.78
	Resting cells in 5% glucose solution, pH 6.4	4.79	25.93	0	11.59	33.98	76.29

[a] According to Bevers (21) and Bevers and Verachtert (22). The *Zymomonas* values are the mean from nine strains tested. All cells were grown on the medium of Gibbs and DeMoss (67).



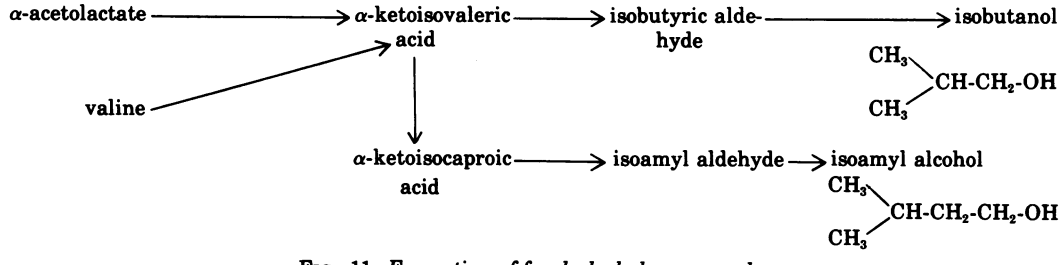


FIG. 11. *Formation of fusel alcohol compounds.*

ate only. Strains of *Z. anaerobia*, examined by Dadds (32), had an absolute requirement for pantothenate. A new isolate from beer required pantothenate, but was also stimulated by biotin (137).

Ca pantothenate and biotin are growth factors for the 38 strains tested by Van Pee et al. (174) (Fig. 12). In 32 strains, they are even the only growth factors required. There are a few strains with some additional requirements, such as 70.9 and 70.10 (vitamin B12), VP2 (*p*-aminobenzoic acid), 7.4 (riboflavin), and the *pomaceae* strain ATCC 29192 (thiamine and riboflavin). VP3 is the most exacting strain, since it requires cyanocobalamine, lipoic acid, folic acid, and riboflavin, in addition to pantothenate and biotin.

In conclusion it may be said that (i) most or all strains require pantothenate and biotin, and occasionally some other growth factors, (ii) re-

TABLE 19. *Changes in growth factor requirements of Zymomonas NCIB 8227*

Report and origin of strain	Reference no.	Growth characteristics	Requirement for:			Lag period (in defined medium)
			Biotin	Lipoic acid	Ca pantothenate	
Bexon and Dawes NCIB	23		+ or +		−	−
Stephenson et al.	158		−	−	+	+
Culture H (maintained at Hull)		No wall growth; no clumping; slow sedimentation				
Culture AB (maintained at Allied Breweries)		Wall growth; clumping; fast sedimentation	−	−	+	−
Culture T (newly obtained from NCIB)		Wall growth; clumping; fast sedimentation	−	−	+	−
Van Pee et al.	174					
NCIB		Clumping; slow sedimentation	+	−	+	
Queensland		No clumping; slow sedimentation	+	−	+	

sults with the same strain differ from one author to another, (iii) upon aging, some strains appear to require pantothenate only, and (iv) differences in growth factor requirements have no taxonomic meaning in *Zymomonas*.

H_2S Formation and Sulfur Metabolism

"Sulfury" aromas are of great technological importance in beer and cider. They are due to H_2S and other unknown compounds (6).

The formation of H_2S by *Zymomonas* has been described by Shimwell (150), Millis (115), Gonçalves de Lima et al. (70), Carr and Passmore (29), Dadds et al. (36), and Richards and Corbey (137). The latter authors claim that H_2S production is easily lost upon subculturing in the laboratory. The production of H_2S may be a stable feature in some strains, e.g., in *Zymomonas* NCIB 8227 and NCIB 8938, which have been subcultured for years and still produced H_2S when tested by Millis (115), Dadds et al. (36), and ourselves.

Forty-two *Zymomonas* strains of our collection do not form H_2S (50). According to Anderson and Howard (5), the main source of H_2S in beer and wort is sulfate (Table 20). On increasing sulfate concentrations from 0 to 500 mg/liter, the amount of H_2S produced increases. Pantothenate has a reverse effect: when its concentration increases from 0 to 40 μg/liter, H_2S formed decreases; higher growth factor concentrations have no additional effect. Zinc ions also stimulate H_2S production (Fig. 13).

Traces of dimethylsulfide and dimethyldisulfide are produced by *Zymomonas* NCIB 8938, B70, and NCIB 8227 (36).

Cellular sulfur is mainly of organic origin (5) (Table 21). Methionine, thiamine, sulfite, sulfate, and cysteine can be used as sources of sulfur in a synthetic medium (35).

Various Features

Presence of catalase. The presence of this enzyme in *Zymomonas* has been demonstrated by Kluyver and Hoppenbrouwers (89), Millis (115), Carr and Passmore (29), and Dadds et al. (36). It is present in all 45 *Zymomonas* cultures of our collection. Belaïch and Senez (17) observed considerable variations in catalase activity of *Zymomonas* NCIB 8938, depending on the composition of the medium. The specific activities of catalase in the minimal and in the synthetic medium were 12 and 5 times higher, respectively, than in the complex medium. The catalase activity remained unchanged after strong aeration for 4 h prior to the assay.

Presence of gelatinase. No gelatinase activity was found in 45 strains of our collection, thus extending the results of Shimwell (151) and Millis (115).

Presence of urease. This reaction was positive but very slow in 24% of the strains of our collection; at least 3 days are needed to show a positive reaction (50).

Oxidase reaction. Dadds et al. (36) reported a negative oxidase reaction in *Zymomonas* NCIB 8938 and B70. Our 45 strains were likewise oxidase negative.

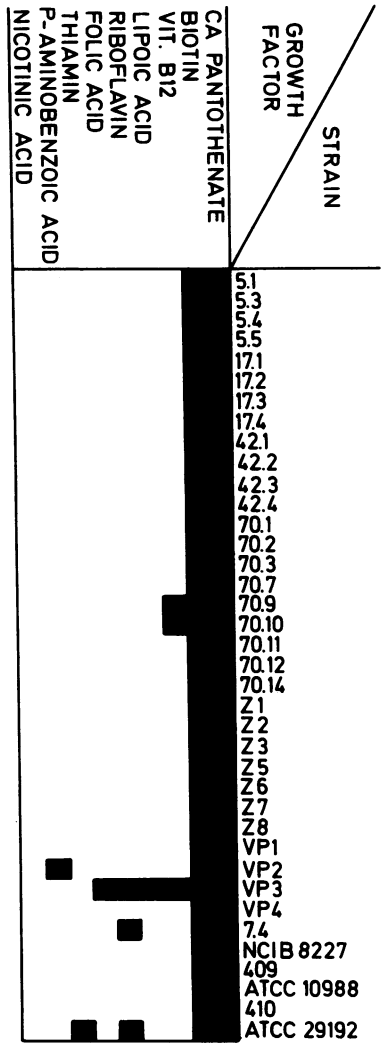


FIG. 12. *Growth factor requirements (dark squares) of Zymomonas strains.*

Hydrolysis of Tweens. Tween 60 and Tween 80 were not attacked (50).

Nitrate reduction. This test was negative in 45 of our *Zymomonas* cultures, a result that agrees with these of Shimwell (151) and of Millis (115).

Formation of indole. Indole was found to be absent by Shimwell (150), Millis (115), and in our 45 *Zymomonas* strains.

CONCLUSIONS AND SUMMARY

Zymomonas cells are gram-negative rods; a minority of the strains are motile, with 1 to 4 polar flagella. These organisms need glucose, fructose, or (for some strains) sucrose in the medium of growth. They are very unusual microorganisms since they ferment these sugars anaerobically by way of the Entner-Doudoroff mechanism, followed by pyruvate decarboxylation. The oxidation-reduction balance between G6P dehydrogenase and triosephosphate dehydrogenase on one hand and ethanol dehydrogenase on the other hand is mediated through NAD^+. Sucrose is converted to some small extent into levan and consumed mainly by a hydrolytic split. Sucrose metabolism seems to be an inducible feature. Sugar fermentation is accompanied by formation of a small amount of

TABLE 20. *Sulfur sources used by Zymomonas NCIB 8227 and Saccharomyces carlsbergensis*[a]

Prepn	H_2S from SO_4 (%)	Cellular S (%) from:		
		SO_4 and methionine	SO_4	Methionine
Zymomonas in:				
Wort	71	56		
Beer	62		35	–[b]
Saccharomyces carlsbergensis in wort	83		21	18

[a] Data from Anderson and Howard (5).
[b] No methionine was detected in beer.

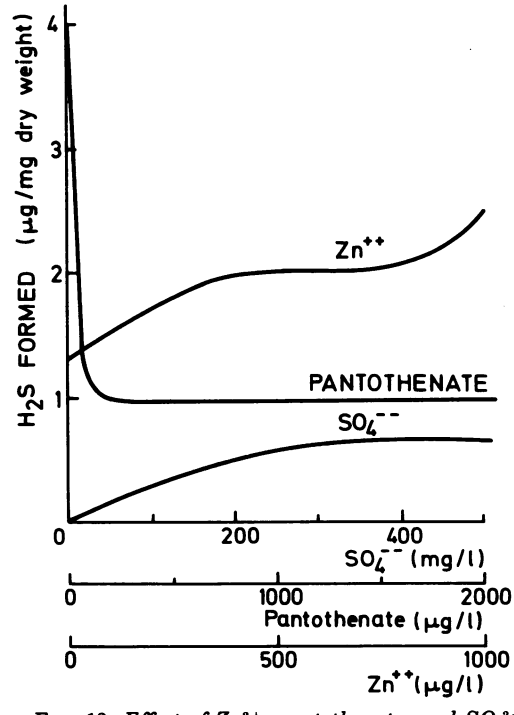


FIG. 13. *Effect of Zn^{2+}, pantothenate, and SO_4^{2-} on H_2S production (data from Anderson and Howard [5]).*

lactic acid, with traces of acetaldehyde and acetoin. The molar growth yield indicates that *Zymomonas* is only about 50% efficient in converting its carbon and energy sources. The growth is partially uncoupled. About 2% of the glucose substrate is the source of about half of the cellular carbon. Several amino acids also serve as carbon sources. Some strains grow only anaerobically; others display various degrees of microaerophily. Apparently, the main effect of oxygen is the oxidation of part of the ethanol to acetic acid. A few enzymes of the Krebs cycle and some cytochromes are also present. Most strains are alcohol tolerant (10%) and grow in up to 40% glucose. None of the 20 amino acids is essential to *Zymomonas* when other amino acids are present. Several amino acids, though, improve growth. This degree of growth stimulation varies from strain to strain. *Zymomonas* produces a small amount of higher alcohols, mainly propanol and isoamyl alcohol, from aspartic acid, homoserine, threonine, and leucine. Most or all strains require pantothenate and biotin; upon aging, some strains appear to require pantothenate only. All strains are oxidase, nitrate, and indole negative. The wide pH range for growth, from 3.5 to 7.5, and the acid tolerance are quite typical.

We have reviewed methods for the detection and isolation of *Zymomonas*, and provided a minimal description for its identification.

Zymomonas is one of the very important ethanol-producing microorganisms in the world. It has been isolated from fermenting agave sap in Mexico, from fermenting palm saps in Zaïre, Nigeria, and Indonesia, from fermenting sugarcane juice in Northeast Brazil. Undoubtedly, they are important contributors to the fermentation of plant saps in many tropical areas of America, Africa, and Asia. These beverages have a great variety of names according to the localities where they are produced. Considerable amounts are sometimes consumed yearly by the local populations. In many cases, these plant wines are produced on a small scale by skilled artisans, but there are some reports of large-scale technological applications, and of therapeutic use. *Zymomonas*, undoubtedly, has a great social and economic importance.

These bacteria also occur in ciders, perries, and beers. So far they have been detected in this habitat in the United Kingdom only. They produce the cider sickness disease, accompanied by much gas formation, change in aroma and flavor, reduction in sweetness, and an unpleasant taste. As a beer contaminant, they cause unpleasant aromas and taste. Recently, *Zymomonas* has also been isolated from ripening honey and bees. *Zymomonas* strains are extremely well adapted to all these ecological niches. Glucose, fructose, or sucrose, amino acids, and growth factors are needed by the bacteria and are provided by the plant juices.

We reviewed critically several proposals made in the literature on the nomenclature and the classification of this genus. A numerical analysis shows that the phenotypical similarity between all the strains is very high; they all form one cluster above 88% S_{SM}. The DNA genome size of all our strains is about 1.5×10^9, expressed as molecular weight. All strains except three from the subspecies *pomaceae* are genetically very similar. The degree of genome-DNA relatedness is at least 76% D in the main group. The cider sickness organism ATCC 29192 has less than 32% genetic similarity with all other zymomonads. The protein electropherograms of all strains except the cider sickness organisms are very similar. There seem to be at least two serological groups, the importance of which is still unclear. Our extensive reexamination of many strains of *Zymomonas* with the above-mentioned modern methods allowed a more realistic classification. Nearly all existing strains of *Zymomonas*, except three, are so similar, in spite of their greatly diverse origins, that they deserve to be united in one taxon, *Zymomonas mobilis* subsp. *mobilis* (Kluyver and van Niel) De Ley and Swings 1976, with the lectotype strain ATCC 10988 = NCIB 8938, and the phenotypic centrotype strain Z6 = ATCC 29192 = NCIB 11999. We proposed a second subspecies, *Z. mobilis* subsp. *pomaceae* (Millis) De Ley and Swings 1976, the cider sickness organism, with the lectotype strain ATCC 29192 = NCIB 11200.

Since all the *Zymomonas* strains, except the cider sickness strains, are genetically and phenotypically almost identical, we suggest that *Zymomonas* is either of recent evolutionary origin, and has not yet had time for genetic diversity, or else it is a genetically very stable genus, in spite of its worldwide distribution.

We also examined the relationship between *Zymomonas* and other genera. *Zymomonas* resembles most closely the acetic acid bacteria, and is closer to *Gluconobacter* than to *Acetobacter*, because of the polar flagella, the occurrence of the Entner-Doudoroff mechanism, the incomplete Krebs cycle, and the ready consumption of glucose. Several other points of similarity were indicated. We suggest that these three genera are of a common phylogenetic origin. This implies that the ancestors of *Zymomonas* were aerobic organisms. Indeed, nearly all organisms with the Entner-Doudoroff mechanism are aerobic. We assume that the aerobic ances-

tors gave rise to the largely anaerobic *Zymomonas* by loss of some Krebs cycle enzymes and perhaps also some electron transport enzymes.

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